Pituitary Adenylate Cyclase-Activation Polypeptide (PACAP) Regulates Feeding in the Rat Striatum and Hypothalamus

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PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE (PACAP) REGULATES FEEDING IN THE RAT STRIATUM AND HYPOTHALAMUS.

by

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ABSTRACT

PITUITARY ADENYLATE CYCLASE ACTIVATING POLYPEPTIDE (PACAP) REGUALTES FEEDING IN THE RAT STRIATUM AND HYPOTHALAMUS.

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The following dissertation focuses on preclinical rodent feeding paradigms that were designed to examine the mechanisms by which the brain regulates caloric (homeostatic) and palatability (hedonic)-driven feeding. Taken together, my findings suggest differentially motivated feeding can, in part, signal through isolated non-overlapping mechanisms in the brain. Furthermore, some of these mechanisms occur in similar neurocircuits that have been implicated in other compulsive behaviors, such as drug abuse.

In an effort to support the argument that binge eating disorder (BED) and substance abuse share similar behavioral and molecular targets, we first demonstrate that the development of BED in rodents is attenuated by both systemic and central administration of a cysteine pro-drug (N-acetylcysteine or NAC) which is a compound that targets the understudied glutamate system and is currently used to treat other disorders that have aspects of compulsion, such as trichotillomania or drug addiction (**chapter II**). Interestingly, NAC-induced hypophagia *is specific* to feeding stimulated by palatability as NAC did not produce any suppression of feeding in animals not maintained under a feeding paradigm that would produce binge behavior.

In addition to studying differentially motivated feeding, a large component of this dissertation examines the mechanisms by which the neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP) regulates feeding in the ventromedial nucleus of the hypothalamus (VMN) as well as the nucleus accumbens (NAc). Our results indicate that PACAP microinjected into the VMN suppresses feeding elicited specifically by food deprivation, as PACAP did not effecting feeding elicited by palatability. Interestingly, in the nucleus accumbens, a brain region important for reward related activity, PACAP suppresses palatably-driven feeding in satiated rats, while not effecting feeding driven by food deprivation (**chapter III**). The opposing behavioral effects of PACAP on feeding propelled the lab to further investigate the mechanism by which PACAP was working in these two regions. In the VMN, we demonstrate that PACAP interacts with leptin signaling as acute blockade of PACAP receptors (PAC1R) in the VMN inhibits the behavioral and molecular actions of leptin (**chapter IV**). In the nucleus accumbens, PACAP attenuates hedonic drive in a site-specific manner and we identified PACAP mRNA expressing striatal afferents originating in the prefrontal cortex (**chapter V**), which is significant as obese individuals display hypoactive medial prefrontal cortex and stimulation of this area decreases calories consumed and body weight. Taken together, the opposing behavioral effects of PACAP emphasize an important point that a signaling mechanism in one brain region can be significantly different in another.

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CHAPTER I

INTRODUCTION

Significance

In 1998, the medical cost alone of obesity in the United States of America was $78.5 billion dollars annually and by 2008 this number rose to a staggering 147 billion annually (Finkelstein, Trogdon, Cohen, & Dietz, 2009). The most costly impact of obesity stems from premature death arising from co-morbid illnesses such as diabetes, high blood pressure, heart attack, and stroke (Allison, Fontaine, Manson, Stevens, & VanItallie, 1999). Currently in the United States, an estimated 30% of the adult population is obese and this number is only expected to rise (OECD, 2013). Taken together, it remains critical to continue to interrogate the mechanisms by which the mammalian brain regulates feeding behavior and how it contributes to the obesity epidemic. By better understanding the central mechanisms governing food consumption, there is a greater chance of identifying and developing novel therapeutic approaches to combat the growing obesity epidemic.

Overview

From single cell to complex multi-cell organisms, the act of seeking and ingesting nutrients is crucial for survival. Therefore, throughout evolution, feeding has become an extremely complex and protected behavior. In mammals, feeding behavior is governed primarily by the brain. It has been well documented that there are a number of *different* brain regions, signaling molecules and genes that regulate feeding behavior (Berthoud &

Morrison, 2008; Lenard & Berthoud, 2008; Rossi & Stuber, 2018). The wide distribution of brain areas and signaling molecules ensures an organism will be able to seek, consume and metabolize calories under a myriad of physiological and behavioral conditions. Evolutionarily, the diffuse complex manner by which the brain regulates feeding can certainly be considered an advantage, but the complexity of this behavior makes it difficult to identify discrete alterations to the brain that drive the pathological development of feeding disorders. With multiple brain sites simultaneously regulating feeding behavior, it becomes difficult to untangle where the site of dysregulation may exist in a pathological feeding state.

To address this challenge, the following dissertation utilizes preclinical rodent feeding paradigms designed to elicit intake stemming from *a specific motivation or drive*. The two motivated feeding states investigated in this thesis include caloric need or **homeostatic hunger** and feeding elicited by the palatability of the food or **hedonic hunger**. Animals that are given access to food after an extended period of food deprivation predictably engage in a large bout of rebound feeding due to homeostatic hunger or the drive to restore caloric resources. Animals given brief intermittent access to a palatable diet will also engage in large bouts of feeding, except this behavior can be driven by hedonic hunger as well as homeostatic hunger. Identifying and understanding the mechanism of feeding behavior that is driven only by a single motive rather than multiple and simultaneous drives will allow us to identify points of vulnerability in feeding systems leading to pathological feeding behavior.

Obesity and binge eating disorder (BED), in part, result from sustained overconsumption, thus leading to excessive weight gain. As this pattern of feeding is

thought to be largely the result of dysregulated hedonic hunger, the studies described in chapter II utilize a limited access binge eating paradigm that produces feeding behavior largely thought to be elicited by hedonic hunger. Animals maintained on this well-accepted preclinical model of BED display large bouts of feeding only for a highly palatable food, which emulates some symptoms observed in human BED (American Psychiatric, 2013). As the frequent excessive binge behavior observed in BED is often compared to other compulsive behaviors such as substance abuse (Johnson & Kenny, 2010; Volkow, Wang, Tomasi & Baler, 2013), we examined whether binge eating behavior could be suppressed by using a compound shown to have therapeutic effects on other compulsive behaviors. In chapter II, we demonstrate that administration of the cysteine prodrug, N-acetylcysteine (NAC) both systemically and centrally curbed BED-like feeding behavior (Hurley et al., 2016b). Interestingly, others have previously demonstrated the therapeutic potential of NAC in treating other compulsive behaviors such as substance abuse or trichotillomania (Grant, Odlaug, & Kim, 2009; McClure, Gipson, Malcolm, Kalivas, & Gray, 2014). These findings reinforce the idea that the compulsive aspect of BED, substance abuse, gambling, or alcoholism may, in part, involve similar maladaptive alterations to brain circuitry that governs reward related behaviors.

In addition to studying the effect of NAC on preclinical rodent models, chapters III-V examine the mechanisms by which the neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP) regulates feeding behavior in the rat ventromedial nucleus of the hypothalamus (VMN) as well as the nucleus accumbens (NAc). Previously, our lab has demonstrated that PACAP microinjected into the VMN potently

suppresses food intake, increases core body temperature and locomotor activity and ultimately decreases body weight (Resch, Albano, et al., 2014; Resch et al., 2011; Resch et al., 2013; Resch, Maunze, Phillips, & Choi, 2014). As these experiments were primarily conducted on animals that had *ad lib* access to standard rat chow, we attempted to explore whether PACAP induced hypophagia in the VMN was specific to homeostatic or hedonic hunger motivated feeding drives. In chapter III, we demonstrate that PACAP microinjected into the male rat VMN only suppressed feeding elicited by homeostatic hunger but not hedonic hunger. Interestingly, in the nucleus accumbens, a region of the brain important for reward processing, PACAP had no effect on homeostatic hunger but did induce hypophagia on hedonic-driven feeding (Hurley et al., 2016a). The distinctive role of PACAP in these two brain regions reinforces the need to avoid broad conclusions or interpretations of compounds or signaling molecules that can overtly regulate behaviors such as feeding. As a result, chapters IV and V are dedicated to further understanding the mechanisms by which PACAP signals in each of these brain regions. Chapter IV demonstrates that acute antagonism of endogenous PAC1R-dependent activity in the VMN blocks the action of leptin on both behavioral and molecular endpoints illustrating an important point that PACAP in the VMN does not regulate feeding behavior independently, but rather in conjunction with more classical metabolic, homeostatic signals such as leptin. Additionally, this dataset demonstrates the potential that central leptin resistance may not be a disruption specifically of the leptin signaling cascade but an integrated signaling mechanism involving PACAP actions. To further characterize the actions of PACAP in the accumbens, chapter V demonstrates PACAP microinjections into the nucleus accumbens broadly decreases the hedonic impact of a

palatable solution and opposes dopamine actions on palatable feeding. Animals pretreated with the dopamine receptor 1 agonist, SFK 81297, blocked PACAP induced hypophagia of palatable feeding suggesting that PACAP and dopamine may interact in this reward center of the brain. Thus, we show that PACAP can effect one type of feeding in the hypothalamus through an interaction with leptin, and regulate a different motivated feeding behavior in the accumbens through interactions with dopamine.

The following introduction will include: i) a description of behavioral similarities between human BED and frequently used preclinical models; ii) a brief outline of human and rodent studies demonstrating the similarities between BED and substance abuse; iii) studies demonstrating the important role leptin and PACAP play in the VMN regulation of energy expenditure and finally iv) a summary of the relevant preclinical studies examining food intake and hedonic perception in the nucleus accumbens.

Preclinical rodent models of binge eating emulate different aspects of BED.

In a study of 1,984 obese individuals seeking weight-loss counseling, approximately 30% of them met the criteria for the diagnosis of BED (Spitzer et al., 1992). Others have found similar trends (Fairburn, 2008; Hudson, Hiripi, Pope & Kessler, 2007) and even demonstrated that BED is associated with severe obesity with BMI measurements greater than 40% (Fairburn, 2008). Currently, the DSM-5 defines BED as recurrent, large meals of highly palatable food that occurs *without* compensatory behavior (i.e. vomiting, laxatives, etc.) observed in other feeding disorders such as bulimia nervosa. Additionally, those who suffer from BED often report a loss of control when bingeing, as well as behavior that is largely thought to be dictated by hedonic

hunger as binge episodes can occur *regardless of energy state* (American Psychiatric, 2013). Thus, BED is a prominent risk factor for potential excess weight gain (Hudson et al., 2010). Interestingly, obese individuals diagnosed with BED display enhanced dopamine release in the dorsal striatum in response to a food stimulus compared to obese individuals not diagnosed with BED (Wang et al., 2011). Enhanced activity of the dorsal striatum is observed in drug addicts and is thought to be responsible for the habitual compulsive component of addiction (Gerdeman, Partridge, Lupica, & Lovinger, 2003). This demonstrates an important point that BED has a unique compulsive component potentially involving the reward center of the brain, in a manner that is not typical of most obese individuals. The following section will illustrate data from different preclinical rodent models that emulate certain aspects of human BED.

The sugar addiction model

Compared to healthy and obese controls, BED patients most often prefer sweet palatable food, which are primarily composed of sugar and over refined-carbohydrates (Davis et al., 2008). The "sugar addiction" preclinical rodent paradigm uses intermittent sugar access in combination with food deprivation to generate BED-like behavior. In brief, this model food deprives rodents for 12 hours and then four hours into the dark cycle animals are given 12-hour access to a sugar solution (either 10% sucrose or 25% glucose) as well as a nutritionally balanced chow (Avena, Rada, & Hoebel, 2006, 2008; Colantuoni et al., 2001). Most notably, animals maintained on this paradigm display large bouts of sugar solution intake, especially during the first hour of access, which the experimenters define as binge behavior (Colantuoni et al., 2001). Animals maintained on the sugar addiction model consumed significantly more sucrose solution compared to

control animals with *ad lib* (continuous) access to the same sugar solution and chow (Avena et al., 2008). Besides recurrent large meals of highly palatable food, animals subjected to this model display heightened states of anxiety and depressive-like phenotypes (Avena et al., 2008). As a result, this model additionally mirrors the psychiatric disorders such as increased, anxiety, depression and most notably, substance abuse that can be similarly observed in individuals diagnosed with BED (Grilo, White, & Masheb, 2009).

Although the sugar addiction model provides several behavioral phenotypes that mimic aspects of human BED, it should be noted that this preclinical model of BED did not produce any significant differences in weight gain compared to control groups. As animals begin to escalate sugar intake they also self-restrict on chow resulting in no net change in total calories (Avena & Hoebel, 2003; Colantuoni et al., 2002). While a lack of weight gain in BED patients has been reported (Spitzer et al., 1993), the majority of BED patients do gain significantly more weight compared to obese control subjects (Ivezaj, Kalebjian, Grilo, & Barnes, 2014). Finally, the sugar and chow being offered 4 hours into the dark cycle and following a 12-hour food deprivation, suggests that although animals display binge behavior in the first hour they have access to the sugar solution, this behavior may also be motivated by homeostatic hunger in addition to hedonic hunger since the cumulative caloric intake is not different between the binge and control group suggesting homeostatic set points are still in control.

Limited access binge model

Unlike the liquid diet in the sugar addiction model, the limited access model primarily uses a highly palatable, high fat diet offered in a sporadic intermittent fashion

as an adjunct to their *ad lib* standard chow (Corwin et al., 1998; Dimitriou, Rice, & Corwin, 2000). Like the sugar addiction model, animals offered limited access to a highly palatable, calorie dense food display large bouts of consumption of the palatable diet, while self-restricting intake of the standard diet offered *ad lib* (Corwin et al., 1998). This self-restriction of standard chow leads to a lack of weight gain, similar to what was reported in the sugar addiction study and is a limitation to understanding the mechanisms responsible for the development in BED *and obesity*. In chapter II, we utilize a similar limited access paradigm to demonstrate that therapeutic approaches used to treat compulsive behaviors such as substance abuse are also effective at suppressing compulsive binge feeding.

Compulsion in BED & substance abuse overlap: Behavioral & molecular evidence

The maladaptive feeding behavior observed in binge eating disorder can culminate into a destructive state similar to drug addiction (Gearhardt, White, & Potenza, 2011; Johnson & Kenny, 2010). As compulsive feeding behavior in BED patients is likely a result of excessive hedonic drive rather than unmet homeostatic needs, there is an overwhelming need to continue examining the parallels between these two motivations, in humans as well as preclinical models (Fisher & Birch, 2002; Marcus & Kalarchian, 2003). The following section will outline both human and rodent data and highlighting a number of similarities between the two disorders.

Human evidence

In a study of 404 patients diagnosed with BED, 73.8% of patients were also diagnosed with at least one other psychiatric disorder in their lifetime and 24.8% of these individuals developed substance abuse (Grilo et al., 2009). This high rate of comorbidity may be due to the loss of control and compulsive aspect of both disorders, which could manifest through similar neuronal adaptations. There are a number of symptoms (Fig 1.1) such as escalation of use, loss of control, social consequences and personal distress that are observed in both disorders (Smith & Robbins, 2013). In addition to these symptoms, chronic substance abuse and BED patients also display similar cognitive deficits effecting executive function, inhibitory control, impulsivity, cognitive flexibility and other complex behaviors regulated by the frontal cortex (Aloi et al., 2015; Boeka & Lokken, 2011; Kelley, Yeager, Pepper, & Beversdorf, 2005; Lyvers & Yakimoff, 2003).

Comorbid Symptoms	*Substance Dependence*	*Binge Eating Disorder*
Escalation of Use	The substance is taken in large amounts or over a longer period than intended.	Eating large amounts of food when not feeling physically hungry.
Loss of Control	There is a persistent desire or unsuccessful effort to cut down or control substance use .	A sense of lack of control during the episodes, e.g., a feeling that one can't stop eating or control what or how much one is eating
Social Consequences	Important social, occupational, or recreational activities are given up or reduced because of use.	Eating alone because of being embarrassed by how much one is eating.
Personal Distress	The substance use is continued despite knowledge of having a persistent physical or psychological problem that is likely to have been caused or exacerbated by the substance.	Feeling disgusted with oneself, depressed , or feeling very guilty after overeating; marked distress regarding being eating; eating until feeling uncomfortably full.

Adapted from Smith & Robbins, 2013 *Biol. Psychiatry*

Figure 1.1 In humans, the symptoms associated with substance dependence appear to mirror many behaviors observed in binge eating disorder.

In addition to behavioral similarities, BED patients reveal abnormalities in a number of well-known reward centers of the brain, which are also highly implicated in the development of substance abuse (Michaelides, Thanos, Volkow, & Wang, 2012). For example, BED patients have diminished activation of cortical regions (ventromedial prefrontal, inferior-frontal, insular) responsible for impulse control in tasks designed to test this behavior (Balodis et al., 2013). Furthermore, BED patients with hypoactive frontal cortices during reward processing were more likely to engage in subsequent binge sessions following treatment (Balodis et al., 2014). Like BED, substance abuse patients (Canterberry, Peltier, Brady, & Hanlon, 2016), pathological gamblers and those diagnosed with obsessive compulsive disorder (OCD) display diminished activation of frontal cortical regions, which demonstrates that the neural adaptations resulting in compulsion can drive a number of different disorders ranging from substance abuse and BED to gambling and OCD (Choi et al., 2012).

Besides the consistent dysfunction in cortical areas the striatum, a subcortical structure, is also a critical region for encoding and driving reward-related behavior further illustrating the similarities that occur between substance abuse and BED (Berridge & Kringelbach, 2015; Kim, Lee, Yun, & Kim, 2017). In humans, body weight gain is associated with increased striatal response to palatable food cues (Stice & Yokum, 2016) a brain region known to robustly respond to substances of abuse and use DA as a primary signal for hedonic experiences. Specifically, following chronic drug administration, addicts display reduction in striatal dopamine 2 (D2) receptor availability (Noble, 2000; Volkow et al., 1990). A similar magnitude decrease in D2 availability can also be observed in obese individuals (Wang et al., 2001). Taken together, human studies of

addicts and BED patients demonstrate that there are a number of overlapping symptoms present in each disorder.

Rodent studies

Animals maintained on a binge eating paradigm display several similarities to models of substance abuse. Behaviorally, animals offered limited access to a highly palatable food source display escalation behavior over time (Corwin et al., 1998), which is also observed in rodent cocaine self-administration models. Additionally, forced abstinence of the binge eating food source produces heightened states of anxiety, aggression and withdrawal-like symptoms, which have been observed in models of drug addiction (Colantuoni et al., 2002; Kantak & Miczek, 1986; Martin, Wikler, Eades, & Pescor, 1963; Schulteis, Yackey, Risbrough, & Koob, 1998; Way, Loh, & Shen, 1969). Interestingly, like humans, rodents maintained on a binge eating paradigm exhibit significant cognitive impairments suggesting potential prefrontal impairment (Chawla, Cordner, Boersma, & Moran, 2017) and similar to the cognitive deficits observed in rodent models of substance abuse. In rodents, both drug and food stimuli result in dopamine release in the striatum (Rada, Avena, & Hoebel, 2005; Wise et al., 1995) as well as decreases in available D2-like receptor binding in the striatum and decreased D2 mRNA levels in animals undergoing binge eating or substance abuse paradigms (Johnson & Kenny, 2010). **The data presented in chapter II builds on the idea that substance abuse and binge eating disorder are linked as we demonstrate N-acetylcysteine, a cysteine prodrug used to treat some compulsive behaviors, has therapeutic effects on a preclinical model of binge eating.**

Pituitary adenylate cyclase-activating polypeptide (PACAP)

As mentioned previously, a major component of this thesis is dedicated to understanding the mechanisms by which PACAP regulates feeding in the brain. PACAP was first isolated in ovine hypothalamic pituitary cells and was named after its ability to stimulate adenylate cyclase (Miyata et al., 1989). PACAP is a member of the secretin-glucagon superfamily of peptides which consists of 9 peptides that play important roles in a number of peripheral and central biological processes (Arimura, 1998; Sherwood, Krueckl, & McRory, 2000). PACAP is 96% conserved over 700 million years of evolution, making it the ancestral molecule for which the other members of the glucagon superfamily were derived. The PACAP precursor, prepro-PACAP (Fig 1.2), undergoes post-translational processing resulting in a biologically inactive PACAP related-protein (PRP) or an active PACAP molecule such as PACAP 1-27 or PACAP 1-38 (Li, Nakayama, Shuto, Somogyvari-Vigh, & Arimura, 1998; Li, Shuto, Somogyvari-Vigh, & Arimura, 1999). Interestingly, both PACAP 1-27 and 1-38 despite being structurally different, show little difference biologically as PACAP 1-27 stimulates adenylate cyclase to similar levels as PACAP 1-38 (Miyata et al., 1990). All PACAP experiments in this dissertation use PACAP 1-38, since this is the predominate form of PACAP in the central nervous system

PACAP gene data:

Species	Chromosomal location	Entrez GeneID	References
human	18p11	116	Hosoya et al., 1992
rat	9q37	24166	Cai et al., 1995
mouse	17E5	11516	Okazaki et al., 1995 Yamamoto et al., 1998

hPACAP precursor (prepro-PACAP):

Adapted from Dickson and Finlayson, 2009 *Pharmacol Ther.*

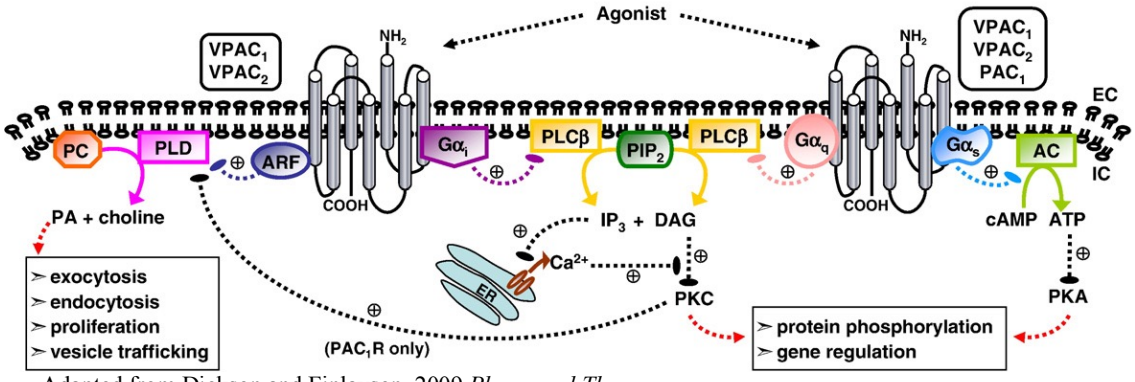
Figure 1.2 The PACAP precursor is 176 amino acids in length and can be processed into PACAP-27 or PACAP-38.

(Ghatei et al., 1993; Hannibal et al., 1995). As PACAP mRNA is widely distributed throughout the brain, this ancient neuropeptide plays a functional role in a number of complex behaviors including, stress, mating, learning, memory, circadian rhythms, cell survival and energy homeostasis (Hammack & May, 2015; Hannibal, Georg, & Fahrenkrug, 2017; Hawke et al., 2009; Lee & Seo, 2014; Morley, Horowitz, Morley, & Flood, 1992; Roberto & Brunelli, 2000; Shintani et al., 2002). Although PACAP has a number of well characterized peripheral effects on insulin & adrenaline secretion, vasodilation, and immunosuppression (Vaudry et al., 2000), the following chapters exclusively examine the mechanisms by which PACAP regulates behavior centrally.

PACAP receptors

As PACAP is 68% homologous with vasoactive intestinal peptide (VIP), these two peptides signal through the same three receptors: PAC1R, VPAC1 and VPAC2. PACAP has much greater affinity for PAC1R compared to VIP (dissociation constant (K_D); PACAP= 0.5nM; VIP>500 nM), whereas PACAP and VIP have similar affinities (dissociation constant (K_D); PACAP & VIP= 1.0 nM) for the VPAC receptors (Dickson & Finlayson, 2009; Vaudry et al., 2000). PAC1R, VPAC1 and VPAC2 are all classified as a B1 subclass of G-coupled protein receptor or GPRCs that couple with Gαs and stimulate adenylate cyclase activity. Besides pairing with Gαs, the PAC1 and VPAC receptors also couple with Gαq and activate a wide variety of intracellular signaling cascades (Fig 1.3; Dickson & Finlayson, 2009). The VIP/PACAP receptors have diverse capabilities in their activation of second messenger systems, which may be due, in part, to the highly alternatively spliced PAC1 receptor (Blechman & Levkowitz, 2013; Dautzenberg, Mevenkamp, Wille, & Hauger, 1999; Kilpatrick, Dautzenberg, Martin, &

Eglen, 1999). A majority of PAC1R splice variants occur in the 3rd intracellular loop of humans, amphibians, fish and rodents that add one or two cassettes of 84 nucleotides (hip or hop1 variants) or 81 nucleotides (hop2 variant) or a combination of the two (Blechman & Levkowitz, 2013). **In chapter III we demonstrate PACAP microinjected into the VMN and striatum produce opposing effects on feeding behavior and cell physiology.**



Adapted from Dickson and Finlayson, 2009 *Pharmacol Ther.*

Figure 1.3 Activation of PAC1R, VPAC1, VPAC2 can stimulate a number activation of secondary messenger systems. In addition to increasing cyclic AMP (cAMP), activation of PACAP receptors can increases phospholipase C, protein kinase C, phosphliipase D and ultimately lead to an increase in intracellular calcium, depending on which G proteins are bound.

Central PACAP regulates different aspects of energy expenditure

Shortly after PACAP was discovered in 1989, it was demonstrated that an intracerebroventricular (ICV) injection of PACAP in mice strongly induced hypophagia (Morley et al., 1992), and was later found to produce similar hypophagic effects in gold fish, chicks, and rats (Matsuda et al., 2005; Mizuno et al., 1998; Tachibana et al., 2003). Although PACAP can signal through the three different receptors reviewed above, antagonizing the PAC1 receptor specifically blocks ICV-PACAP induced hypophagia

(Mounien et al., 2009). Interestingly, global knockout (KO) of PACAP, PAC1R or VPAC2 receptors results in a lean animal, which conflicts with expectations based on the ICV pharmacological studies (Adams et al., 2008; Asnicar et al., 2002; Jamen et al., 2000). As PACAP is widely distributed in the brain, and throughout the body, the loss of PACAP or PAC1R may have variable effects on feeding behavior that is dependent on the specific brain region. However, global KO of PACAP or PAC1R does result in early lethality, suggesting the PACAP signaling is critical to survival and healthy development (Gray, Cummings, Jirik, & Sherwood, 2001; Otto et al., 2004).

Exogenously applied PACAP is thought to produce changes in feeding behavior primarily through actions in the hypothalamus, which is supported by the extensive PAC1R expression in several hypothalamic nuclei and the abundance of PACAP protein in this brain region. In fact, the concentration of PACAP in the hypothalamus is approximately 25 times greater than PACAP concentrations in the cerebral cortex (Hannibal et al., 1995; Hashimoto et al., 1996; Resch et al., 2011). Within the hypothalamus, immunohistochemical analyse of PACAP nerve fibers demonstrate that almost all nuclei within the hypothalamus receive dense PACAP innervation, primarily from either the paraventricular nucleus of the hypothalamus or the ventromedial nucleus of the hypothalamus (Hannibal, 2002; Nomura et al., 2000). Additionally, PACAP in the hypothalamus is responsive to energy states such that mice maintained on a calorie-dense diet for two months show significant increases in hypothalamic PACAP mRNA, whereas a 48 hour fast decreases mRNA levels (Hawke et al., 2009).

Besides direct effects on feeding behavior, PACAP-related manipulations also have significant effects on the autonomic nervous system and metabolism. Previous

studies have demonstrated that ICV injections of PACAP increase core body temperature, oxygen consumption and locomotor activity in rodents (Hawke et al., 2009; Masuo, Noguchi, Morita, & Matsumoto, 1995; Pataki, Adamik, Jaszberenyi, Macsai, & Telegdy, 2000). PACAP/PAC1R KO mice have elevated circulating levels of corticosterone, cholesterol, ketones, and triglycerides as well as lower than usual levels of liver glycogen, which contributes to undernourished pups with poor life expectancies (Gray et al., 2001; Otto et al., 2004). Interestingly, elevating colony room temperature greatly increased longevity of PACAP KO pups emphasizing an important role for PACAP in regulating body temperature (Gray, Yamaguchi, Vencova, & Sherwood, 2002). PACAP also has an important role in maintaining glucose homeostatic as PACAP, PAC1R and VPAC2 KO mice display impaired glucose tolerance and insulin hypersensitivity (Adams et al., 2008; Asnicar et al., 2002; Tomimoto et al., 2008). Thus, the diverse array of functions regulated by central and peripheral PACAP demonstrates that it is intimately tied to energy expenditure and metabolism. Examining specific hypothalamic subgroups, our lab has previously demonstrated that an acute microinjection of PACAP directly into the ventromedial nucleus of the hypothalamus (VMN) strongly suppresses feeding, stimulates core body temperature, locomotor activity and ultimately decreases body weight (Resch et al., 2011; Resch et al., 2013; Resch, Maunze, et al., 2014). While the role of endogenous PACAP in a specific aspect of feeding behavior has still not been confirmed, blockade of PAC1R alone in the VMN during the dark cycle does not produce any significant alteration to feeding behavior whereas, antagonizing the PAC1R during the light phase significantly increases feeding behavior. These results suggest that

PACAP's endogenous hypophagic actions occur primarily during the light phase when animals do not typically eat.

The VMN regulates energy state in part through the actions of PACAP & leptin.

The ventromedial nucleus of the hypothalamus (VMN) is located on the ventral surface of the brain on either side of the third ventricle (Paxinos & Watson, 2007). Over the years several studies have demonstrated that the VMN plays a critical role in maintaining a homeostatic set point for energy expenditure. For example, electrical stimulation of the VMN results in decreased feeding behavior in rats while ablation of the VMN produces an obese animal (Beltt & Keesey, 1975; Bray & York, 1979; Hetherington & Ranson, 1939). The VMN contain a number of receptors known to regulate energy homeostasis such as the hypothalamic growth hormone secretagogue-receptor (GHS-R), PAC1R and the long form of the leptin receptor, LepRb (Bennett et al., 1997; Hannibal, 2002; Hashimoto et al., 1996). Leptin receptors are abundantly expressed throughout hypothalamic nuclei including the VMN (Elmquist, Bjorbaek, Ahima, Flier, & Saper, 1998).

Central leptin signaling in the VMN

Leptin is an adipocyte-derived hormone that is secreted by fat cells and acts both peripherally and centrally to inhibit feeding behavior as well as regulate energy expenditure and autonomic homeostasis (Jequier, 2002; Rosenbaum & Leibel, 2014). As the production of leptin in fat cells is correlated with the size of the fat cell (Considine et al., 1995; Funahashi et al., 1995), obese individuals have chronically elevated leptin

levels that is hypothesized to lead to both peripheral and central leptin resistance (Adeyemi & Abdulle, 2000). In rodents, systemic administration of leptin rapidly decreases food intake and body weight (Campfield, Smith, Guisez, Devos, & Burn, 1995; Halaas et al., 1995; Pelleymounter, Cullen, Baker, et al., 1995; Stephens et al., 1995; Weigle et al., 1995). ICV microinjections of leptin confirm that leptin actions to suppress food intake and decrease body weight are centrally mediated (Schwartz et al., 1996). Interestingly, lesioning the VMN produces desensitization to central leptin administration in rats (Choi, Sparks, Clay, & Dallman, 1999) suggesting a functioning VMN is necessary for central leptin induced hypophagia. *In situ* hybridization by others and from our lab (Fig 4.2) demonstrate that the leptin receptor (LepRb) is expressed in the VMN (Elmquist et al., 1998; Mercer et al., 1996). The leptin receptor is a cytokine receptor that signals in the VMN through the Janus Kinase 2 (JAK2)-signaling transducer and activator of transcription 3 (STAT3) cascade and upon activation produces an increase in STAT3 phosphorylation (P-STAT3) which subsequently leads to gene expression (Jiang, Li, & Rui, 2008). An ICV injection of leptin directly activates cells in the arcuate, dorsomedial and ventromedial hypothalamic nuclei by increasing P-STAT3 immunoreactivity (Hubschle et al., 2001). In addition to increasing P-STAT3 levels, leptin signaling also stimulates immediate early gene expression of c-fos in the VMN further demonstrating that leptin activates hypothalamic neurons (Elmquist, Ahima, Maratos-Flier, Flier, & Saper, 1997), Additionally, direct microinjection of leptin into the VMN induces hypophagia more potently than an ICV injection (Jacob et al., 1997), which is consistent with previous studies demonstrating that electrical stimulation (i.e. activation) of the VMN decreases food intake (Beltt & Keesey, 1975). Although a large

amount of work has been done to characterize the mechanisms by which leptin exerts its hypophagic effects in the arcuate nucleus (Baver et al., 2014), less is known about leptin's functions in the VMN. Interestingly, leptin and PACAP when microinjected in the VMN nearly identical physiological and behavioral effects.

Evidence for PACAP and leptin interaction in the VMN

In addition to the dense expression of PACAP in the VMN, steroidogenic factor-1 (SF-1) is also abundantly expressed in this nucleus (Meek et al., 2016). Targeted knockdown of SF-1 or leptin receptors specifically in SF-1 neurons in the VMN produce an obese animal (Dhillon et al., 2006; Majdic et al., 2002; Zhao et al., 2004). Thus, leptin mediated signaling in SF-1 neurons in the VMN is critical to maintaining energy expenditure. Interestingly, a recent publication demonstrated that SF-1 and PACAP mRNA are also co-localized in the VMN (Hawke et al., 2009). We have demonstrated PACAP and PAC1R are co-expressed in the VMN suggesting the possibility that exogenously applied PACAP is inducing hypophagic effects by activating PAC1R on LepRb/SF-1/PACAP/PAC1R expressing cells of the VMN (Fig 4.2). In support, Hawke and colleagues demonstrate that an ICV injection of the PAC1R antagonist, PACAP 6-38, into the third ventricle blocks ICV leptin induced suppression of food intake, stimulation of thermogenesis and decreased body weight (Hawke et al., 2009). The behavioral and physiological similarities that result from individual administration of either PACAP or leptin into the VMN and the evidence that PACAP 6-38 interferes with intraventricular administration of leptin suggests that both peptides could interact in the VMN regulate food intake and energy expenditure. **To address this gap in knowledge,**

chapter IV of this dissertation evaluates to what degree leptin and PACAP interact in the VMN on a behavioral and molecular level.

The nucleus accumbens is a critical site for the regulation of reward related behavior.

The nucleus accumbens, which can be anatomically and behaviorally dissociated into a core and shell region (Zaborszky et al., 1985), is located in the ventral compartment of the striatum and is a critical site for regulation of reward related behaviors (Berridge & Kringelbach, 2015). As the nucleus accumbens receives dense dopaminergic projections from the ventral tegmental area (Wise & Bozarth, 1984), the primary output cells of the nucleus accumbens (GABAergic medium spiny neurons or MSNs) can be sorted into two distinct populations based on whether they express D1-like or D2-like receptors as previous studies have estimated that only 5% of MSNs express both receptor types (Bertran-Gonzalez et al., 2008). Furthermore, MSNs can be distinguished based on their efferent targets as well. D1-like receptor expressing MSNs predominately project to the substantia nigra and ventral pallidum (Bocklisch et al., 2013; Kupchik et al., 2015), whereas D2-like receptor expressing MSNs largely project to the ventral pallidum (Wall, De La Parra, Callaway, & Kreitzer, 2013). Rewarding stimuli, such as food or drug, increase extracellular dopamine in the nucleus accumbens and promote approach behavior (Rada et al., 2005; Wise et al., 1995). As the nucleus accumbens receives dense glutamatergic afferents from the hippocampus, amygdala and prefrontal cortex (Floresco, Todd, & Grace, 2001; McFarland, Lapish, & Kalivas, 2003; Morgane, Galler, & Mokler, 2005; Stuber et al., 2011), dopamine concentrations in the nucleus accumbens act to alter the sensitivity of these MSNs to glutamatergic drive from

these upstream structures (Surmeier, Ding, Day, Wang, & Shen, 2007). Interestingly, chemogenetic activation of D1 neurons in the nucleus accumbens, increases feeding behavior in rats, while inactivation of these neurons, attenuates feeding behavior (Zhu, Ottenheimer, & DiLeone, 2016). **As PACAP microinjected into the nucleus accumbens attenuates hedonic hunger, chapter V of this dissertation includes preliminary studies examining whether pharmacological manipulation of D1-like receptors alters PACAP-induced changes palatable food intake.**

Inactivating caudal accumbens regulates hedonic perception & feeding behavior

In chapter III of this dissertation, we demonstrate that PACAP suppresses palatable-feeding in the nucleus accumbens by inhibiting neuronal activity. In addition to our work, others have demonstrated that PACAP microinjected into nucleus accumbens potently suppresses cocaine-primed reinstatement in rats (Hess et al, *in prep*), suggesting that PACAP actions in the accumbens broadly function to curb compulsive behaviors. Interestingly, pharmacological inactivation of the caudal dorsomedial nucleus accumbens, produced a significant suppression in feeding behavior in addition to stimulating defensive escape behavior (Reynolds & Berridge, 2001). The anatomical site targeted by Reynolds & Berridge is immediately adjacent to sites targeted in our own nucleus accumbens studies. One could posit that activation of this region of the accumbens is necessary to encode rewarding stimuli or promote the ingestion of palatable food, whereas inactivation of this area may encode aversive associations. In addition, studies have shown that ingestion of foods high in sugar or fat produce a significant increase in immediate early gene expression in the nucleus accumbens core but not in the

shell demonstrating that the nucleus accumbens core is sensitive to palatable components of food (Dela Cruz et al., 2015). Taste reactivity paradigms have been used to measure hedonic perception of a palatable tastant and is a method to quantifying "liking" (Ho & Berridge, 2014). Human infants, monkeys and rodents display highly conserved orofacial responses to tastants that can be categorized as the animal either "liking" or "disliking" a certain stimuli (Grill & Norgren, 1978). Interestingly, inactivation of the caudal accumbens, the same region that elicits hypophagia following inactivation, suppresses "liking" of a 1% sucrose solution, while simultaneously increasing "disliking" responses (Ho & Berridge, 2014). However, it is still not clear whether PACAP in the accumbens suppresses palatable food intake by altering the hedonic perception of the rewarding food. **To address this gap in our understanding, chapter V of this dissertation examines the effect of NAc-PACAP on the hedonic perception of a 1% sucrose solution using the taste reactivity paradigm.**

System xc- implications in PACAP & the development of addictive behaviors.

A number of studies demonstrate compulsive drug seeking behavior, in part, is dependent on glutamatergic afferents from the prefrontal cortex to the nucleus accumbens core (Cornish & Kalivas, 2000; Di Ciano & Everitt, 2001; Park et al., 2002). Compulsive drug seeking behavior, followed by withdrawal from the substance of abuse, results in decreased extracellular glutamate levels in the nucleus accumbens (Hotsenpiller, Giorgetti, & Wolf, 2001; Pierce, Bell, Duffy, & Kalivas, 1996). Interestingly, restoring extracellular basal glutamate levels with cysteine prodrug, N-acetylcysteine (NAC), blocks cocaine primed reinstatement (Baker et al., 2003). NAC increases glutamate

concentrations through activation of the astrocytic cystine-glutamate antiporter, system xc-, whereas antagonizing system xc- with (S)-4-carboxyphenylglycine (CPG) blocks NAC induced increase in extracellular glutamate concentrations (Baker et al., 2003; Ye, Rothstein, & Sontheimer, 1999). In brief, system xc- exchanges one molecule of extracellular cystine for one molecule of intracellular glutamate (Sato, Tamba, Ishii, & Bannai, 1999). Systemically administered NAC can deliver cysteine into the brain (Griffith, 1999; Meister, 1985), which can effectively drive the non-vesicular release of glutamate in the nucleus accumbens. **Since PACAP receptors are expressed by glia (Ashur-Fabian, Giladi, Brenneman, & Gozes, 1997), and recent studies demonstrate that PACAP application *directly drives* the activity of system xc- (Kong et al., 2016; Resch, Albano, et al., 2014), chapter V conducts studies to determine if PACAP-mediated suppression of hedonic hunger is in part dependent on system xc- in the accumbens.**

Summary

The following dissertation is comprised of preclinical animal paradigms designed to elicit specific motivated feeding states. As a number of studies suggest binge eating disorder and substance abuse share the element of compulsion, in chapter II we examine if a therapeutic approach used to treat other compulsive behaviors is effective at curbing the development of binge eating behavior.

Previously, our lab has demonstrated that PACAP microinjected into the ventromedial nucleus of the hypothalamus (VMN) profoundly suppresses food intake as well as induces several other notable changes in energy expenditure (Resch et al., 2011; Resch et al., 2013; Resch,

Maunze, et al., 2014). However, it was still not clear if VMN-PACAP manipulations regulate feeding behavior driven by a specific type motivation. Therefore, chapter III utilizes a novel feeding paradigm designed to separate homeostatic and hedonic hunger within the same animal to examine in detail the manner in which PACAP regulates feeding in the VMN. Furthermore, in chapter III we tested whether or not PACAP-mediated regulation of feeding behavior was ubiquitous in all brain structures by examining PACAP mediated regulation of feeding behavior in the striatum. In chapter IV, we examine the mechanism by which PACAP interacts at behavioral and molecular levels with leptin in the VMN, while in chapter V we study the impact of PACAP in the nucleus accumbens on hedonic contributions, in addition to possible interactions with dopamine and system xc-. Collectively, these findings demonstrate that central PACAP signaling regulates different aspects of feeding depending on *where* in the brain PACAP is acting and which specific motivation or drive to eat, homeostatic (i.e. leptin in the VMN) or hedonic (i.e. dopamine in the nucleus accumbens), is activated.

CHAPTER II

N-ACETYLCYSTEINE (NAC) DECREASES BINGE EATING IN A LIMITED ACCESS RODENT MODEL

INTRODUCTION

Binge eating disorder (BED) is one of the most prevalent eating disorders in the United States and contributes to the obesity epidemic that is currently endangering the health of approximately 30% of all Americans (Hudson, Hiripi, Pope, & Kessler, 2007; OECD, 2013). Feeding behavior in BED is characterized by rapid consumption of large amounts of highly palatable food that occur in very short periods of time. Additionally, those suffering from BED often eat until feeling uncomfortably full and will consume food whether they feel the need to eat or not (American Psychiatric, 2013). Feeding stimulated by the palatability of the food source rather than the need to fill caloric stores is referred to as ***hedonic hunger*** (Lowe & Butryn, 2007) and in healthy, lean individuals hedonic hunger is experienced in moderation (i.e. dessert) with little to no negative effect. However, in BED patients, this type of palatability-driven hunger appears to be pathologically dysregulated.

Compared to individuals with obesity, patients with BED displayed significantly higher levels of craving toward sweets, which was not observed in the obese population (White & Grilo, 2005). This was determined using the Food Craving Inventory (FCI), which is a self-reporting mechanism for monitoring a patient's food craving for a particular food source (White, Whisenhunt, Williamson, Greenway, & Netemeyer, 2002). In a separate study, BED patients engaged more frequently in nocturnal feeding bouts of high fat, high sugar foods whereas non-BED controls did not engage in this behavior

(Greeno, Wing, & Marcus, 1995). These studies reinforce the idea that obesity and BED are in fact different. Obesity can manifest through a number of *non-hedonic* related endocrine diseases such as hypothyroidism and Cushing's syndrome (Garrapa, Pantanetti, Arnaldi, Mantero, & Faloia, 2001; Sanyal & Raychaudhuri, 2016; Tiryakioglu et al., 2010), whereas BED appears to manifest through specific dysregulation of the neuronal mechanisms governing hedonic hunger. To further emphasize this point, an fMRI study using a Monetary Incentive Delay Task (MIDT) as a measure of reward processing demonstrated that in individuals who were treated for BED but then relapsed to bingeing behavior showed diminished recruitment of reward related brain areas (ventral striatum, frontal gyrus) as compared to BED patients who were treated for BED but did not relapse (Balodis et al., 2014). This suggests that reward related circuitry, which is involved in the consumption of highly palatable foods, may be the source of dysregulation when it comes to binge eating disorder. This illustrates the importance to continue interrogating the neural underpinnings by which the brain regulates hedonic hunger in order to increase the likelihood of developing more effective therapeutic approaches to combat binge eating behavior and reduce obesity rates.

Although drug addiction has been shown to involve abnormal glutamatergic signaling in the nucleus accumbens (Kalivas, LaLumiere, Knackstedt, & Shen, 2009), there are very few therapeutic approaches that directly target this system. One compound that does target the glutamate system, the cysteine prodrug N-acetylcysteine (NAC), has been shown to reduce many forms of compulsive behaviors ranging from addiction to trichotillomania (Amen et al., 2011; Grant et al., 2009). NAC works centrally by increasing the activity of the cystine-glutamate antiporter, system xc- (McClure et al.,

2014). NAC does this by increasing extracellular cystine concentrations, which drives the 1:1 exchange of extracellular cystine for intracellular glutamate through system xc-. Increasing extrasynaptic glutamate tone via non-vesicular release can, in turn, modify glutamate release and subsequent receptor function (ie synaptic signaling) in regions implicated in motivated or compulsive behaviors including hedonic motivated eating (Bridges, Lutgen, Lobner, & Baker, 2012; Lin & Pratt, 2014). In support, system xc- has been shown to be an effective target to inhibit cocaine-induced behaviors and reinstatement in pre-clinical studies (Baker et al., 2003). Because of the *compulsive* aspect of binge eating, an overeating behavior analogous to drug abuse, we examined the potential for systemic or intraventricular administration of NAC to alter binge eating in rodents.

To determine if NAC can effectively curb binge eating behavior, we utilized a pre-clinical model of binge eating in which rodents are permitted daily limited-access to a highly palatable food as an adjunct to their *ad lib* standard chow in order to recapitulate some of the characteristics of human binge eating disorder (Corwin et al., 1998). Animals maintained on this paradigm display rapid bouts of feeding specifically of palatable food indicating an excessive hedonic feeding drive.

MATERIALS & METHODS

Animals

Male Sprague-Dawley rats (225-250g; 53-58 days old; Harlan; Indianapolis, IN) were housed individually under a 12 hour light/dark cycle (2PM: Lights OFF; 2AM: Lights ON) with *ad lib* access to a Harlan standard diet (8604 formula) for the duration of

all experiments. Intake was monitored daily via a BioDAQ Food Intake Monitor (Research Diets, New Brunswick, NJ) or by pre-weighing food bins prior to and after experimental sessions. Body weights were also recorded daily. All procedures using animals were approved by the Marquette University Institutional Animal Care and Use Committee.

Cannulation Surgery & Microinjections

Animals were anesthetized with a ketamine/xylazine/acepromazine (77:1.5:1.5 mg/ml/kg; i.p.) cocktail and placed in a stereotaxic apparatus. A 26 gauge unilateral guide cannula (Plastics One; Roanoke, VA) was placed into a lateral ventricle and secured to the skull via an acrylic resin. The stereotaxic coordinates were anterior/posterior, -0.8 mm from bregma; medial/lateral, ± 1.5 mm from midline; dorsal/ventral, -3.0 mm from the surface of the skull, whereas the injector extended 0.5mm further beyond the tip of the guide cannula. Stereotaxic coordinates were based on The Rat Brain in Stereotaxic Coordinates, 6th Edition (Paxinos & Watson, 2007).

Limited access binge-eating model

Animals with *ad lib* access to standard chow (Harlan 8604 formula; SC; 32% of total kcal protein, 54% of total kcal carbohydrate, 14% of total kcal fat; 3.0 kcal/g) were additionally allowed daily limited access (30 min/day) to a highly palatable western diet (WD; Research Diets; New Brunswick, NJ; 17% of total kcal protein, 43% of total kcal carbohydrate, 41% of total kcal fat; 4.7 kcal/g) shortly after lights out (30 minutes). Chronic systemic (i.p..; 90mg/kg) or intermittent intraventricular (ICV; 10ug/5ul)

injections of NAC were administered 30 minutes prior to gaining limited access to WD similar to the procedures used in previous work examining the pre-clinical therapeutic potential of systemic and centrally administered NAC in attenuating cocaine relapse behavior in rodents (Yonatan M. Kupchik et al., 2012).

Chronic systemic N-acetylcysteine: Limited access binge

Animals (n=12-17/treatment) received chronic intraperitoneal (i.p.) injections of either vehicle (saline) or N-acetylcysteine (60, 90 or 120 mg/kg) 30 minutes prior to gaining limited access (30 min/day) to a pre-weighed bin of WD for 7 days. Animals receiving 90 mg/kg were maintained on a binge paradigm for an additional 7 days, because NAC at this dose significantly suppressed western diet consumption after the first 7 days.

Central N-acetylcysteine: Limited access binge

After one week to recover from guide cannula surgery, animals (n=6-8/treatment) were placed on the limited access binge paradigm for 7 days during which NAC was injected into the cerebral ventricles on alternating days as to not associate the western diet with the central injection and to avoid producing significant cell damage due to repeated daily injections. On days 1, 3, 5, and 7 animals received central infusions of NAC (10µg/5µl) while control animals received an equal volume of vehicle (saline); microinjections were administered 30 minutes prior to WD access. Animals were given daily access (30min/day) to WD to avoid associating the novel food bin with injections.

Acute systemic N-acetylcysteine: ad lib SC

Animals (n=7-8/treatment) acclimated to the BioDAQ food monitoring system received intraperitoneal injections of vehicle (saline) at the onset of the dark cycle for one week to habituate the animals to the mild stresses of the injection. Following the habituation period, animals were either injected with vehicle or NAC (90 mg/kg;) at the onset of the dark cycle and the animals' feeding behavior were monitored 1 and 3 hours post injection.

Acute systemic N-acetylcysteine: Limited access binge

Animals (n=6/treatment) were entrained for 14 days to the limited access binge paradigm as described above combined with a daily intraperitoneal injection of vehicle (saline) 30 minutes prior to gaining access to WD to habituate animals to the stresses of the injection. On the 15th day, all animals received an injection of NAC (90 mg/kg) 30 minutes prior to western diet access followed by measurement of post-injection food intake.

Conditioned Taste Aversion

A separate group of animals were habituated to restricted water access (1HR/day) and systemic injections (i.p.) of saline 30 minutes prior to water access for 1 week. Subsequently, animals were separated into three treatment groups (n=5/group): saline, NAC (90 mg/kg; IP), or LiCl (2%; i.p.) and each offered two bottles containing a 0.15% saccharin solution for 24 hours then returned to their previous restricted water conditions. Two days later, fluid consumption was measured in animals provided a two-bottle choice

test with one bottle containing 0.15% saccharin solution and the other containing water for 1 hr.

Progressive ratio

To introduce the testing environment, rats (n=4/treatment) were food deprived for 24 hours and placed overnight in an operant chamber that allowed animals to press a lever to receive a 45 mg pellet of high fat diet (Bio Serv; Flemington, NJ). For the subsequent three days, non-food deprived animals received a vehicle injection (saline) 30 minutes prior to being placed in the operant chamber for 30-minute lever access to the high fat diet pellets at the onset of the dark cycle. Animals were then put through two progressive ratio tests on separate days as described previously (Richardson & Roberts, 1996), where the animal received vehicle prior to one session and NAC (90 mg/kg) prior to the other session.

Statistics

Data are presented as means ± standard error of the mean, and were analyzed statistically (Sigma Plot 11; SystatSoftware Inc.; San Jose, CA) by analysis of variance (with repeated measures when appropriate) or student's t-test. Fischer LSD analysis was used for all post-hoc group comparisons. P values less than 0.05 were considered statistically significant.

RESULTS

Animals given daily limited access to a highly palatable western diet (WD) in addition to *ad lib* standard chow (SC) display binge-like behavior (rapid consumption of calories) over an extended time course (Fig 2.1A). By day 14, a significantly larger percentage of the animal's daily intake consists of the WD consumed during the 30 minute period vs the 24 hour *ad lib* access to standard chow (Fig 2.1B; $p<0.05$). Interestingly, animals given limited access to WD consumed significantly more calories compared to animals given *ad lib* access to SC or WD in a similar 30-minute time period (Fig 2.1C; $p<0.05$). Taken together, the rodent limited-access binge paradigm produces a state of rapid consumption that appears to be driven by palatability. In an effort to attenuate this binge eating, we administered the cysteine prodrug N-acetylcysteine (NAC) which effectively attenuated other compulsive behaviors such as drug seeking in humans (Amen et al., 2011) and drug-reinstatement in rodents (McClure et al., 2014).

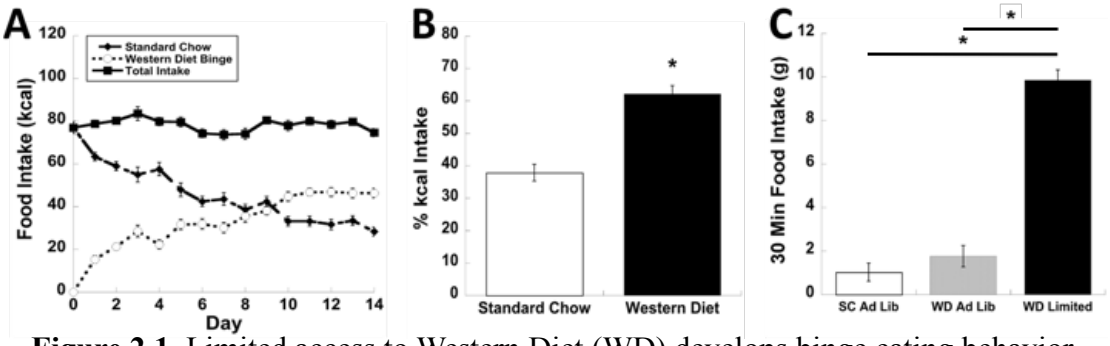


Figure 2.1 Limited access to Western Diet (WD) develops binge eating behavior. (A) Comprehensive view of Standard Chow (SC, *ad libitum*), WD (30 min/day) and cumulative food intake over a 14 day period. (B) Percent kilocalorie intake of WD on day 14 is significantly increased compared to SC intake. (C) Animals given limited access to WD will consume significantly more in a 30 minute period as compared to animals with *ad libitum* access to either SC or WD. Data expressed as mean ± SEM. * = P < .05.

Daily injections of NAC (60, 90 or 120 mg/kg; i.p.) 30 minutes prior to gaining access to the palatable meal decreased WD intake at all three doses (Fig 2.2). Although only animals receiving the 90 mg/kg dose displayed statistically significant suppression of WD consumption compared to saline injected animals (Fig 2.2B; Tx $F(1,186)= 4.517$, $p=.043$). As a result, the experiment using the 90 mg/kg dose was repeated and extended to 14 days to evaluate the efficacy of NAC under chronic conditions (Fig 2.3).

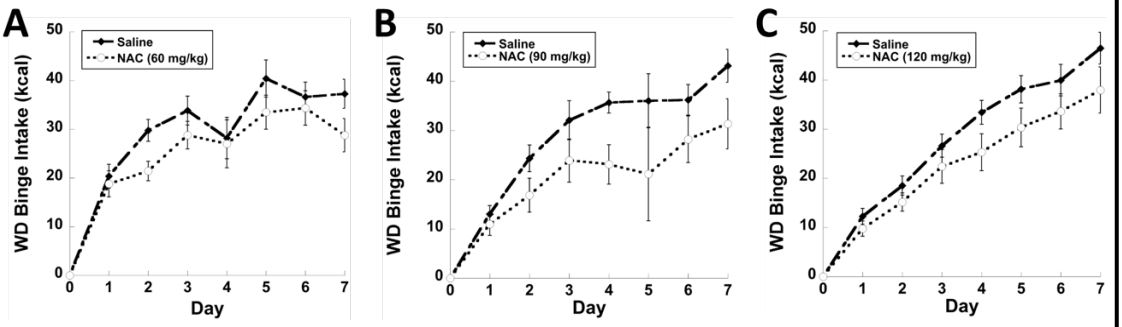


Figure 2.2 Dose response study of daily N-acetylcysteine (NAC) intraperitoneal (i.p.) administration on WD (30 min/day) binge. (A,C) NAC administered at 60 and 120 mg/kg respectively, did not significantly attenuate WD consumption compared to control. (B) NAC administered at 90 mg/kg demonstrated a significant suppression of WD intake. Data expressed as mean ± SEM.

Daily systemic injections of NAC (90 mg/kg; i.p.) or vehicle (saline) were administered 30 minutes prior to limited access (30 min/day) to WD as an adjunct to *ad lib* SC for 14 days. Animals treated with NAC consumed significantly less WD (Fig 2.3A; treatment $F(1,405)= 5.478$; $p=0.03$), and significantly more SC (Fig 2.3B; treatment $F(1,405)= 4.893$; $p<0.04$) over the 2 week study. As expected, NAC treated animals displayed a significantly larger percentage of daily kilocalorie ingested from SC and a concomitant reduction in WD intake compared to vehicle treated rats by the 14th day of experimentation (Fig 2.3C; SC % intake $t= 2.463$, $DF=27$, $p=0.020$; WD % intake

t= -2.463; DF=27, p=0.020). The compensatory increase in SC consumption paired with the attenuation in WD consumption resulted in no significant change in total calories consumed or body weight gain (Fig 2.3D-E). In a separate study, NAC administered centrally every 48 hours (prior to WD access) at the onset of dark via intraventricular (ICV) injections displayed a similar suppression in the escalation of WD consumption over a 7 day period (Fig 2.3F; treatment X time F(6, 97)= 2.281; p=0.45).

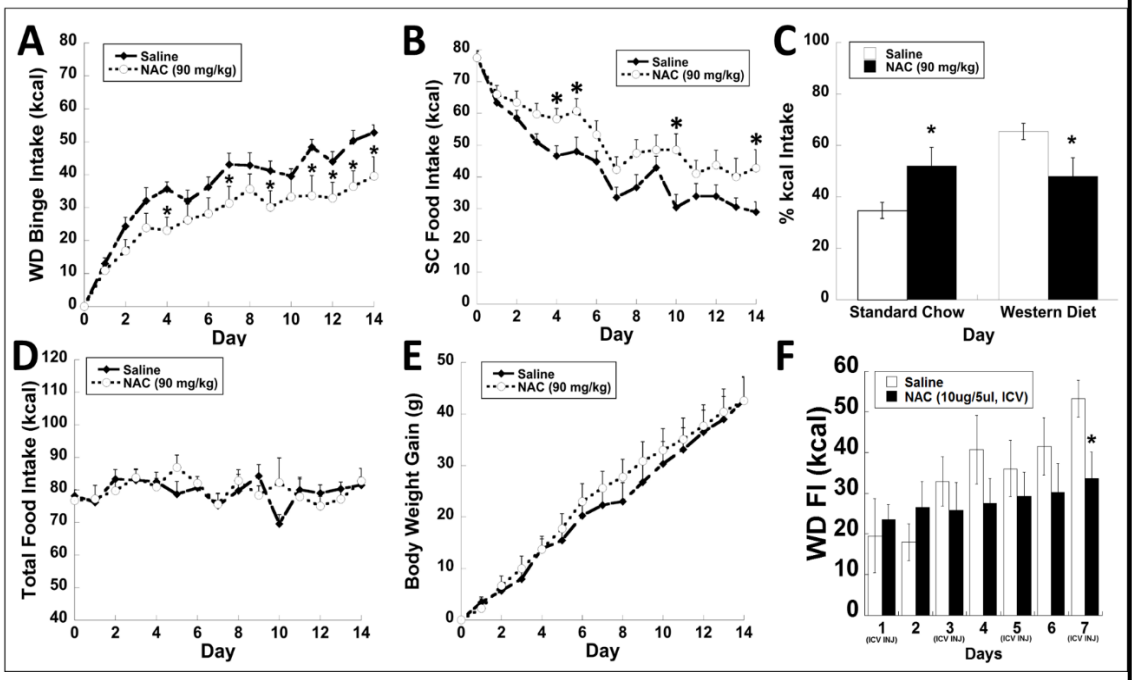


Figure 2.3 System (90mg/kg; i.p.) and central (10µg/5µl) NAC alters binge behavior produced by daily limited access to a palatable food. **(A)** NAC treated animals ate significantly less WD (30 min/day) compared to saline injected control animals. **(B)** Subsequently, NAC treated animals ate significantly more SC over the duration of the study. **(C)** Percent kilocalorie intake of SC on the 14th day is significantly higher for the NAC treated group, while WD consumption was significantly decreased compared to the control group. **(D,E)** There is no significant difference in cumulative food intake or body weight gained between the two experimental groups. **(F)** In a separate study, animals received infusions (NAC or saline; ICV) 30 minutes prior to accessing WD, central administration of NAC significantly suppressed WD consumption over the 7 day study. Data expressed as mean ± SEM. * = P < .05 compared to control.

The hypophagic effects of NAC appear to be specific to palatable WD consumption as it did not significantly alter the feeding behavior of animals maintained on *ad lib* SC only (Fig 2.4A). Moreover, there were no delayed effects on standard chow consumption at the later time points of 6 or 24 hours after administration of NAC (data not shown), which indicates the action of systemic NAC on food intake is specific to palatable food binge. Additionally, a conditioned taste aversion study revealed that suppression of WD by NAC was not due to NAC-induced malaise, which was observed in LiCl treated animals (Fig 2.4B; water vs saccharine t= -43.717, DF=8, p<0.001).

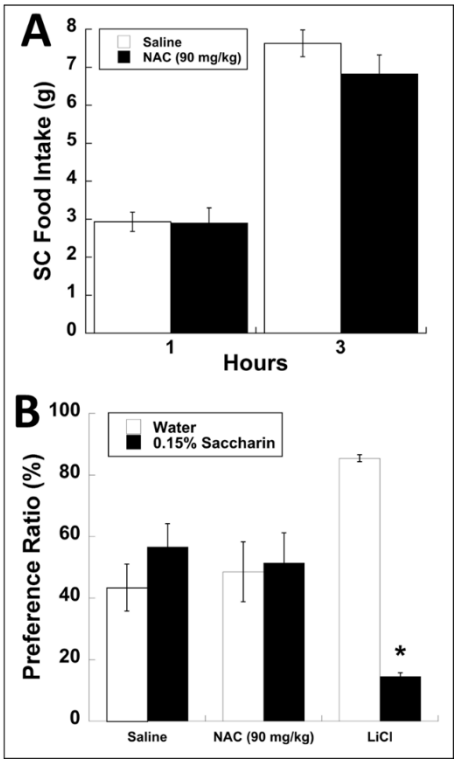


Figure 2.4 Systemic administration of NAC (90mg/kg; i.p.) does not alter *ad libitum* SC consumption or produce any conditioned taste aversion. **(A)** Animals maintained on *ad libitum* SC and not subjected to a limited-access binge paradigm show no changes in feeding behavior measured 1 and 3 hours post i.p. injection. **(B)** Saccharin preference ratio (%) of control (saline), NAC and LiCl (2% bw) treated animals produced only a significant suppression of saccharin consumption in the LiCl treated group. Data expressed as mean ± SEM. * = $P < 0.05$ compared to controls.

To determine if NAC was effective in acutely blocking established binge behavior, animals were entrained to the limited WD binge paradigm for 14 days and given daily injections of vehicle (to acclimate to the injection process) and then administered NAC (90 mg/kg; i.p.) on day 15. Interestingly, acute administration of

NAC did not produce any significant change in WD intake compared to the vehicle injection on day 14 (Fig 2.5).

To better understand if NAC alters the reinforcing property of a high-fat diet reward, animals were placed in a progressive ratio paradigm whereby the animals could lever-press to receive a high-fat diet pellet. Within-subject analysis revealed interesting trends in both "lever presses" and "rewards received" with systemic NAC appearing to suppress both categories, but neither reaching statistical significance (Fig 2.6A-B; Lever presses *paired t-test* t= 2.194, DF= 5, p=0.080; HFD rewards *paired t-test* t= 2.236; DF=5, p=0.076).

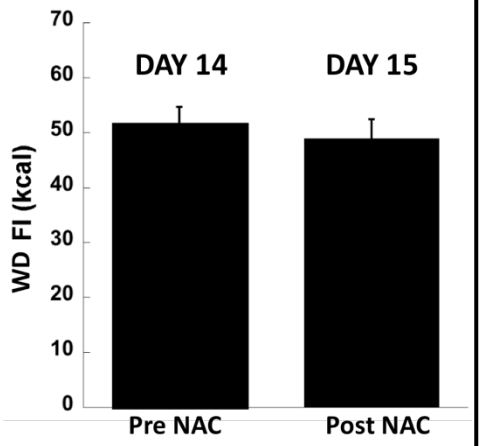


Figure 2.5 Systemic administration of NAC (90 mg/kg) following a 14 day binge paradigm does not suppress WD consumption. Animals were given saline injections (i.p.) for 14 days while receiving limited access to WD (30 min/day), on the 15th day animals were injected with NAC prior to WD access. Data expressed as mean ± SEM.

DISCUSSION

Male rats provided daily limited access to a highly palatable food source display some of the behavioral similarities observed in human binge eating disorder. After 14 days of this feeding schedule, animals consumed nearly 60% of their daily intake in the brief period (30 min) of access to the western diet (Fig 2.1B). As the DSM-5 defines BED as the consumption of large quantities of highly palatable food in a brief period of time, we believe the limited access model accurately mimics this aspect of human BED

(American Psychiatric, 2013). Interestingly, studies using a similar design have reported that this model does not induce weight gain compared to control animals (Corwin et al., 1998). This is likely because as the animal escalates daily intake in the western diet they limit intake in the SC thereby, maintaining consistent cumulative intake levels (Fig 2.1A). We can conclude that although this feeding design produces some similarities to human binge eating, there are important differences, namely the lack of weight gain. These results strongly suggest that while this feeding paradigm does appear to engage a hedonic hunger drive, other motivations such as homeostatic drives to eat are still engaged and perhaps act to limit the ability to clearly interpret this behavioral data.

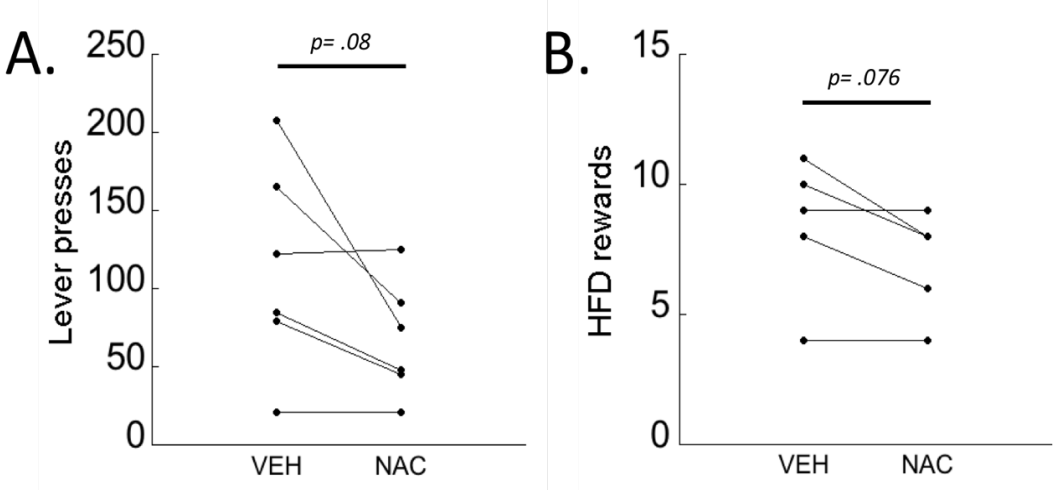


Figure 2.6 Acute systemic NAC (90 mg/kg; i.p.) on high fat diet progressive ratio. **(A)** NAC did not affect cumulative lever presses compared to vehicle injected data. **(B)** Animals treated with NAC did not consume rewards differently compared to vehicle injected groups. Data expressed as within-subject individual data points in each test.

As a number of parallels can be drawn between substance abuse and BED, we examined whether the cysteine prodrug N-acetylcysteine (NAC) can attenuate binge

feeding behavior observed in a rodent limited-access feeding model as it has been shown in previous studies of compulsive behaviors in both humans and animals. Chronic systemic N-acetylcysteine significantly attenuated binge eating of a WD over a 14 day study (Fig 2.3A-C). Although chronic NAC administration did not alter total caloric intake or weight gain (Fig 2.4D;2.4E), systemic or central administration of NAC (Fig 2.3F) was sufficient to reduce intake of highly caloric and palatable food without inducing malaise (Fig 2.4B). Additionally, NAC did not alter feeding behavior in animals only given *ad lib* SC, suggesting NAC induced hypophagia is specific to excessive hedonic feeding behavior (Fig 2.4A). Interestingly, acute systemic NAC was ineffective at blocking binge behavior in an animal already entrained to the binge paradigm (Fig 2.5), whereas acute NAC did produce an interesting non-significant trend in high fat diet progressive ratio (Fig 2.6). A potential interpretation for why NAC was ineffective at blocking an established 14-day binge behavior may result from a well-established learned association of the highly palatable food source, such that a single treatment of NAC may be ineffective at overcoming this highly learned rewarding stimuli. All of our data related to this binge paradigm would suggest that NAC is only effective when administered across experimental days when learning or establishing the palatable nature of a food is developing. Future studies could examine binge-entrained animals administered daily NAC for at least two weeks to determine whether there is any effect of chronic NAC on established binge behavior.

The selectivity of NAC to only regulate the development of binge eating behavior is quite notable as it suggests that system xc- activation does not impact overall caloric homeostasis but, instead, may lessen the hedonic drive for food. NAC, which has been

demonstrated to drive the activity of the cystine-glutamate antiporter, system xc-, promotes non-vesicular glutamate release and consequently impacts synaptic glutamate signaling, which is critical to the development and maintenance of a wide variety of compulsive behaviors (Baker, Xi, Shen, Swanson, & Kalivas, 2002; Kalivas, Lalumiere, Knackstedt, & Shen, 2009). Under conditions of long-term use of drugs of abuse there is a reduction in system xc- activity in the nucleus accumbens, which has been proposed to contribute to the pathological glutamate signaling contributing to addiction (Baker et al., 2003). Further illustrating the importance of glutamate signaling in compulsive behaviors, NAC treatment reduces cocaine-induced behaviors in animals and cravings and or use of cocaine and tobacco in humans (Amen et al., 2011; Baker et al., 2003; Knackstedt et al., 2009). Although pursuing effective treatments for compulsive eating disorders will require further understanding of the neural mechanisms that underlie hedonic drive, the effectiveness of NAC to reduce binge eating of a highly caloric palatable food is consistent with its effect in studies of compulsive behaviors such as drug taking, hair pulling and skin picking, and suggests that system xc- is a potential therapeutic target for the treatment of compulsive feeding disorders (Amen et al., 2011; Grant et al., 2009; Odlaug & Grant, 2007).

CHAPTER III

PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE (PACAP) REGULATES HOMEOSTATIC- AND HEDONIC-INDUCED BINGE EATING.

INTRODUCTION

A fundamental barrier to treating obesity is the challenge associated with understanding the specific origin of excessive feeding behavior. Until this knowledge is gained development of potential new treatments based on signaling mechanisms will be relegated to trial and error. Discrete forms of obesity, binge eating, or other eating disorders could differentially stem from pathological changes in feeding circuitry driven by homeostatic needs (e.g., homeostatic hunger-driven feeding) or hedonic motivations for highly palatable foods (e.g., hedonic hunger-driven feeding) (Lowe & Butryn, 2007; Lowe & Levine, 2005). However, isolating a specific anorexigenic substance that can suppress a particular feeding drive has been difficult to determine because feeding in many preclinical models likely involves multiple feeding drives. This is particularly problematic with paradigms that compare the consumption of standard chow and highly-palatable food in combination with food deprivation. For example, in the limited-access binge model, subjects have *ad lib* access to standard chow in conjunction with brief access to a highly palatable food, which promotes binge eating (Corwin, 2004; Corwin & Hajnal, 2005; Czyzyk, Sahr, & Statnick, 2010). While *ad lib* access to standard chow should limit homeostatic-driven feeding, animals show self-induced food deprivation with reduced consumption of the devalued standard chow (Corwin & Buda-Levin, 2004). The potential that a confluence of homeostatic and hedonic drives exists in this model is

evident by the observation that daily caloric intake and body weight remain stable in this paradigm despite the addition of the high-caloric food (Bake, Morgan, & Mercer, 2014). In the current studies, we modified this approach by restricting access to both diets in order to promote conditions whereby homeostatic-driven consumption of standard chow resulted in satiety prior to providing subjects access to highly palatable food. By doing this, homeostatic and hedonic drives are more clearly separated, which enabled us to examine the cellular and molecular components of each feeding drive.

Using this new model of binge eating, we first sought to characterize the cellular or regional contributions to homeostatic- and hedonic-driven feeding. Initially, we examined the impact of hypothalamic ventromedial medial nuclei (VMN) activation on feeding primarily driven by homeostatic or hedonic feeding drives. Although the VMN have historically been viewed as satiety centers regulating feeding behavior (King, 2006), it is unknown if the VMN-satiety signal also gates feeding stemming from other distinct drives (e.g., palatable-driven feeding). In addition, we targeted a subregion of the nucleus accumbens (NAc), which has been principally linked to hedonic drives; the degree to which the NAc regulates other motivations to eat including homeostatic-based feeding is less well-studied (Baldo & Kelley, 2007; Baldo et al., 2013; Johnson & Kenny, 2010). Each of these experiments is important because human obesity can stem from either abnormal homeostatic feeding or, over consumption of highly palatable foods even in the absence of homeostatic need (Boggiano, 2016). Hence, these and future studies have the potential to identify drive-specific circuitry, a discovery that could help narrow attempts to outline the neural basis for unique forms of obesity.

An additional objective of the current study was to examine whether a single anorexigenic substance could modify either the activity of NAc- and VMN-related circuitry through homeostatic- or hedonic-feeding drives. Recently, we found that intra-VMN administration of pituitary adenylate cyclase-activating polypeptide (PACAP) markedly suppressed feeding and reduced body weight even in fasted animals via the PAC1R receptor subtype (Resch et al., 2011; Resch et al., 2013). Of the three PACAP receptors, PAC1R is primarily involved in the hypophagic properties of intra-VMN PACAP whereas the contribution of VPAC1 and VPAC2 are not (Resch et al., 2013). While the VMN express an abundant amount of PACAP mRNA, retrograde tracing has revealed numerous extra-hypothalamic efferents including PACAP containing projections from the medial amygdala and lateral parabrachial (Resch et al., 2013). In the NAc, similar retrograde studies show PACAP containing efferent projections to the NAc such as the medial prefrontal cortex (Fig 5.3). PACAP is a highly conserved neuropeptide that is often expressed in glutamatergic neurons and has been primarily implicated in neurohormone signaling, learning and memory, and neurodegenerative responses (Pellegri, Magistretti, & Martin, 1998; Zhou et al., 2002). Thus, it represents an interesting molecular candidate since prior studies have shown that this neuropeptide is capable of activating and inhibiting ionotropic glutamate receptors (Macdonald et al., 2005; Toda & Huganir, 2015). In support, our lab has demonstrated that PACAP's anorexic actions in the VMN likely augments glutamate signaling by potentiating NMDA receptors (Resch, Maunze, et al., 2014) whereas, PACAP in the NAc inhibits evoked firing in medium spiny neurons (Hurley et al., 2016a). Hence, the capacity for PACAP to produce bidirectional changes in excitatory signaling may position this poorly understood

anorexigenic peptide to inhibit NAc-related circuitry and suppress palatable-driven feeding while stimulating VMN-related circuitry to restrict homeostatic-driven feeding.

MATERIALS & METHODS

Animals

Male Sprague-Dawley rats (Harlan; Indianapolis, IN) weighing 350-400 g, were housed individually in either a BioDAQ feeding system, a computer automated data acquisition system that records food intake measurements using an algorithmic load cell technology (Research Diets, New Brunswick, NJ) or standard hanging wire cages under a 12:12 light/dark cycle. Feeding was measured via the BioDAQ system or by weighing food bins before and after experimental sessions (including spilled food). Body weights were collected daily. All animal procedures were approved by the Marquette University Institutional Animal Care and Use Committee.

Diets

We used Harlan standard chow (SC; #8604; 32% protein, 54% carbohydrate, 14% fat; 3.0 kcal/g) or a palatable western diet (WD; #D12079B; Research Diets; New Brunswick, NJ; 17% protein, 43% carbohydrate, 41% fat; 4.7 kcal/g). When indicated, standard chow was flavored with either vanilla, almond (0.05% pure vanilla extract, 0.05% imitation almond extract; The J.R. Watkins Co; Winona, MN) or vehicle (water).

Cannulation Surgery and microinjections

Surgery: Animals were anesthetized with ketamine/xylazine/acepromazine (77:1.5:1.5 mg/ml/kg; i.p.). 26-gauge bilateral guide cannulae (Plastics One; Roanoke VA) were stereotaxically placed 2-3 mm above the ventromedial nuclei (VMN; anterior/posterior, −2.5 mm from bregma; medial/lateral, ± 0.6 mm from midline; dorsal/ventral, −6.2 mm from surface of the skull) or the nucleus accumbens (NAc; anterior/posterior, +1.6 mm from bregma; medial/lateral, +2.2 from midline; dorsal/ventral, -4.8mm from surface of the skull) and secured to the surface of the skull (Paxinos & Watson, 2007). Afterwards, brains were collected, immediately frozen and embedded in OCT for analysis of cannula placement. 30 μm thick sections were Nissl stained and only those with correct placements were included in the studies (Fig 5).

Microinjections: Pituitary adenylate cyclase activating polypeptide (PACAP; 50pmol/0.25 μl/side; California Peptide Research, Napa, CA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA; 74.5ng/side; Tocris Bioscience, Minneapolis, MN); baclofen+muscimol (106.8ng /5.7 ng/side; Tocris Bioscience, Minneapolis, MN) or saline (vehicle) were microinjected into the VMN (0.25 μl/side) or NAc (0.5 μl/side) over a two minute period (using a syringe pump) in gently restrained awake animals followed by an additional minute to prevent backflow.

Restricted feeding

At the onset of dark, animals (n=12 total) were entrained (1 week/regimen) to various restricted feeding durations (2, 3, 4 or 6 hours/day in BioDAQ) using only SC. During the remaining 22, 21, 20, or 18 hours animals did not have access to food. Body

weights were recorded daily. In addition to the restricted feeding groups, animals (n=6/group) fed SC and WD *ad libitum* served as control groups for feeding and body weight measurements.

Two-meal model (M1-M2)

Rats (n=12/group) were entrained to consume their daily SC intake in a 2-hour period after the onset of the dark phase (Meal 1; M1). After establishing consistent feeding patterns and weight gain (40-50 kcal/2hr; body weight gain 2-3 grams/day), animals were offered a short 15 min meal (Meal 2; M2) of either SC or WD (n=6/group) approximately 30 minutes following M1 for 7 days before experimentation. Food intake and body weight measurements were recorded in an additional group (n=6/group) of rats that were *ad lib* fed either SC or WD as control groups.

In separate studies, animals were entrained to the two-meal model (M1-M2) for 5 days before undergoing VMN or NAc cannulation surgery. VMN microinjections of vehicle (n=9-10/group), PACAP (n=7/group), AMPA (n=6/group) or baclofen+muscimol (n=3/group) were separately administered approximately 30 minutes prior to either M1 or M2. Similarly, NAc microinjections of vehicle (n=9-10/group), PACAP (n=9/group), baclofen+muscimol (n=9/group) or AMPA (n=3/group) were administered approximately 30 minutes prior to M1 or M2.

Slice electrophysiology

In collaboration with Dr. Q.S. Liu at the Medical College of Wisconsin, rats were anesthetized by isoflurane inhalation and decapitated. Coronal slices (250 μm; n=6-7 slices/brain region) containing the VMN and the NAc were cut using a vibrating slicer

(VT1000S, Leica) at 4°C with a sucrose-based solution containing the following: 220 mM sucrose, 25 mM $NaHCO_3$, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 0.5 mM $CaCl_2$, 7 mM $MgSO_4$, and 10 mM glucose. The slices were recovered in a sucrose-NaCl-based solution containing the following: 68 mM sucrose, 78 mM NaCl, 25 mM $NaHCO_3$, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM glucose for 30 min at room temperature. The slices were then transferred to artificial cerebrospinal fluid (ACSF) containing the following: 125 mM NaCl, 2.5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 1.25 mM NaH_2PO_4, 25 mM $NaHCO_3$, and 10 mM glucose. The slices were maintained in ACSF for at least 1 hour before electrophysiology recordings. All solutions are saturated with 95% O_2 and 5% CO_2.

Whole-cell or cell-attached recordings were made from the VMN and NAc using patch-clamp amplifier Multiclamp 700B under infrared-differential interference contrast (DIC) microscopy. The VMN is an egg-shaped region located in the mediobasal hypothalamus adjacent to the third ventricle, and the NAc is an area around the optic nerve about 200 μm from the edge of the anterior commissure. Data acquisition was performed using DigiData 1440A digitizer (Molecular Devices). Glass pipettes (4-6 MΩ) were filled with an internal solution containing (in mM): 140 potassium gluconate, 5 KCl, 10 HEPES, 2 $MgCl_2$, 0.2 EGTA, 2 MgATP, 0.3 Na_2GTP and 10 Na_2-phosphocreatine (pH 7.4 with KOH). Signals were filtered at 2 kHz and sampled at 10 kHz. Spikes were driven by current injections from -60 to 300 pA. PACAP (100 nM) was added to the brain slices after the membrane potential was stable and a baseline measurement (control) of spontaneous activity and spike firing followed by application of PACAP to obtain within cell treatment effects. Glutamate receptor antagonist CNQX (10

μM) and $GABA_A$ receptor blocker picrotoxin (50 μM) were present throughout all physiological recordings. Recordings were performed at 32 ± 1°C using an automatic temperature controller (Warner Instrument).

Corticosterone (B) Radioimmunoassay

In a separate group of animals offered *ad lib* SC (n=12) or restricted SC access (2hr/day at the onset of the dark cycle; n=12) for two weeks, half were sacrificed at the onset of the dark cycle (prior to eating), and the remaining half sacrificed 2 hours into the dark cycle or after the restrict feeding session. Plasma B was measured from trunk blood using a radioimmunoassay (MP Biomedicals, Santa Ana, CA).

Statistics

Data are presented as means ± standard error of the mean and analyzed by ANOVA (with repeated measures when appropriate) or Student's t-test. Fisher LSD analysis was used for post-hoc group comparisons using Sigma Plot 11 software (Systat Software Inc.; San Jose, CA). $p<0.05$ = statistical significance.

RESULTS

In order to develop a feeding paradigm that produced fully-satiated animals, we measured the total calories consumed during periods of 2, 3, 4 or 6 hours (HR) of restricted feeding in one minute bins (Fig 3.1A). Animals maintained on 6 HR daily access to SC ate significantly more cumulative kilocalories compared to animals maintained on 2, 3, or 4 HR access (Fig 3.2B; RESTRICT FED $F(3,46)= 17.332$, $p < 0.001$). This is likely because with 6 HR access the animal had enough time to become

satiated and hungry again within the same restrict-fed session. This is illustrated in figure 3.2C which demonstrates animals with only 2 hours access essentially engage in one long continuous acceleration to an extremely satiated state, whereas animals with 6 HR access eat less at the start, take a long break, and start eating again near the end of the 6 HR session. Taken together, with 2 HR access producing the greatest state of satiety in the shortest period of time we used this restrict-fed schedule moving forward in future experiments.

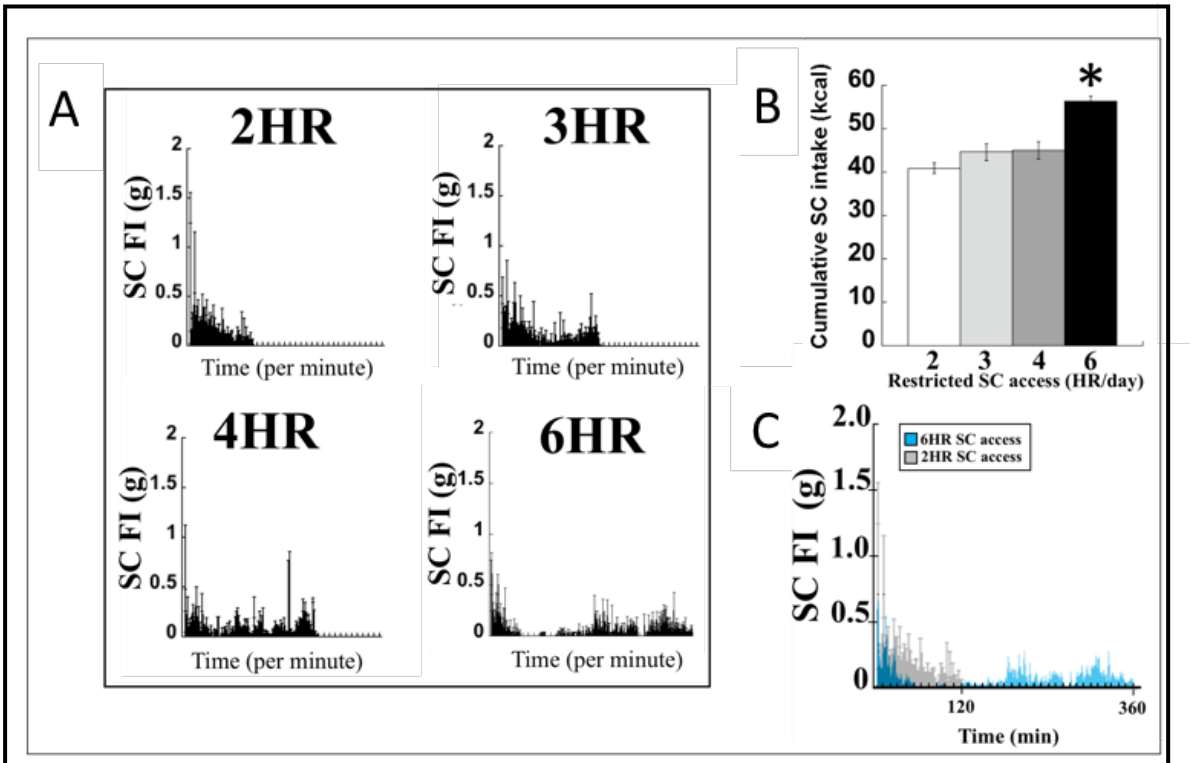


Figure 3.1 Rodents maintained on restricted feeding schedule display large bouts of feeding motivated by homeostatic hunger. **(A)** Grams per minute intake of animals maintained on 2, 3, 4, or 6 hours daily access to standard chow (SC) at the onset of the dark cycle. **(B)** Animals offered 6 hours daily access consume significantly more cumulative kilocalories compared to animals with 2, 3 or 4 hours. **(C)** Comparison of 2HR feeding pattern versus animals given 6HR daily access. Data presented ± SEM, * = $P < 0.001$.

Two-meal model (M1-M2) and Restricted Feeding

The two-meal model tested food consumption in satiated vs. hungry rats. After entrainment to a 2 hour SC meal (M1), animals were offered a second meal (M2; 15 minutes) consisting of either SC or WD (Fig 3.2A). Animals consuming WD during M2 (SC-WD) consumed significantly more total daily calories than rats receiving SC (SC-SC) (Fig 3.2A; DIET $F(1,183)= 78.428$, $p<0.001$; DIET X TIME $F(7,183)= 13.279$, $p<0.001$). Moreover, figure 3.2A shows SC-WD fed animals consumed more calories than animals provided SC (58.6 ± 0.5 Kcal) and WD *ad libitum* (74.9 ± 1.4 Kcal) demonstrating that 2 hour restricted feeding or SC-SC resulted in approximately 25% reduction in daily caloric intake compared to a SC *ad lib* fed animals and that SC-WD animals consumed more calories than *ad lib* WD fed rats. By day 12, animals consumed as many calories from WD during the 15-min M2 as was consumed during the 2 hour M1 (3.2B; $p<0.001$) and as a result gained significantly more body weight than rats receiving SC for M2 (Fig 3.2C; DIET X TIME; $F(6,150)= 32.983$; $p<0.001$). Notably, this increase was significantly greater than even *ad lib* WD fed rats. A significant difference in body weight gain was evident by the third presentation of WD for M2 compared to the SC-SC group ($p<0.001$; Fig 3.2C). In addition, SC-WD animals gained body weight faster than animals maintained on *ad lib* SC or WD (Fig 3.2C). To determine if the two-meal model was a product of novelty, we offered SC made novel by flavoring with either vanilla or almond extract (or control) during M2. There were no differences in M2 intake over three days access to flavored SC (Fig 3.2D; SC vs. almond $p=0.714$; SC vs. vanilla $p=0.902$; almond vs. vanilla $p=0.807$), which contrasted the marked increase in palatable WD intake over the same time period (Fig 3.2D; WD vs. SC, almond or vanilla $p<0.001$).

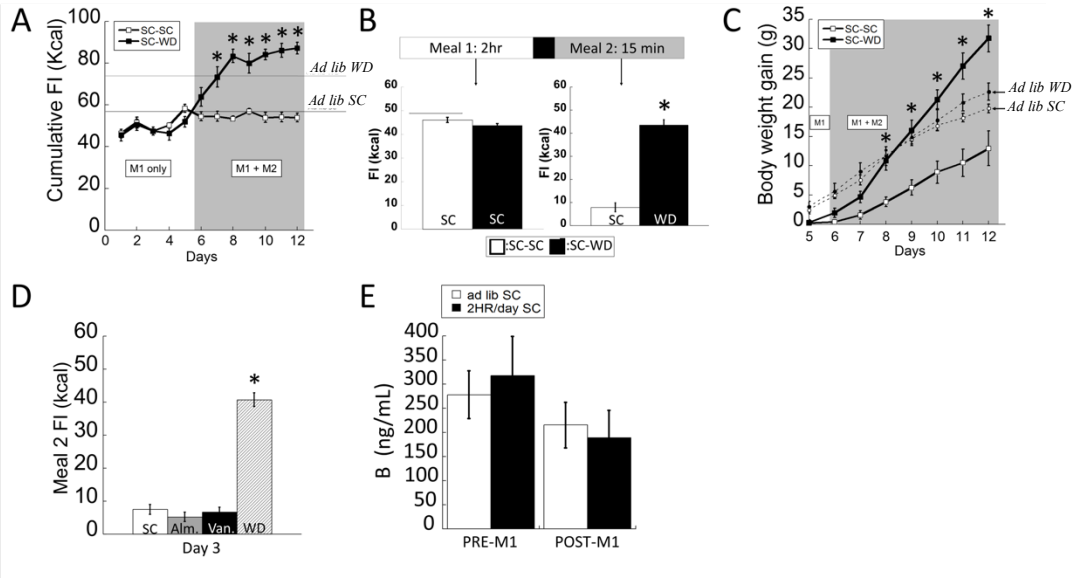


Figure 3.2 The two-meal paradigm maintained animals on restricted SC (standard chow) intake (M1; 2hr/day) followed by access to a second meal (M2; 15 min/day) of SC (M1(SC)-M2(SC)) or a highly palatable diet (WD) shortly after M1, (M1(SC)-M2(WD)). **(A)** SC-WD displayed higher total food intake (FI) levels than SC-SC animals (and *ad lib* fed SC or WD animals). **(B)** By day 12, satiated rats offered WD (SC-WD) consumed more calories than animals offered SC (SC-SC). **(C)** Animals consuming WD (SC-WD) during M2 gained significantly more weight than animals offered SC (SC-SC) and gained more than *ad lib* fed SC and WD animals. **(D)** Food intake levels of SC flavored with almond (Alm.) or vanilla (Van.) during M2 did no differ compared to unflavored SC and were significantly lower than WD. **(E)** Plasma B (corticosterone) levels in *ad lib* and restrict fed animals before M1 and after the onset of dark and 2 hours into the dark cycle (after M1). Data expressed as mean ± SEM. * = $p<0.05$.

We and others have shown that animals entrained to 2 hours of restricted feeding show normal circadian rhythmicity and low basal and normal peak levels of corticosterone (B; < 3 μg/dl and >20 μg/dl, respectively) suggesting that they were not chronically stressed (Choi, Wong, Yamat, & Dallman, 1998; Krieger, 1980). In support, we confirmed that circulating B levels did not differ between *ad lib* and 2 hour restrict fed animals (before and after their meal) during a period of peak B activity (Fig 3.2E; FEEDING REGIMEN $F(1,23)= 0.017$, $p=0.915$; FEEDING REGIMEN X TIME

F(1,23)= 0.302, p=0.589).

VMN microinjections

Intra-VMN PACAP (Fig 3.6A for anatomy) administered prior to M1 produced a significant reduction in SC consumption during M1 compared to non-injected (No INJ) and vehicle injected animals (Fig 3.3A; p<0.001 for both). Intra-VMN AMPA administration also significantly suppressed consumption of SC during M1 (Fig 3.3A; AMPA vs No INJ or vehicle, p<0.001; baclofen+muscimol vs No INJ, p=0.148; vs vehicle, p=0.282) indicating that PACAP and AMPA produced similar behavioral actions in the VMN. Surprisingly, there were no differences in the calories consumed during M2 of either SC or WD when PACAP was administered prior to M1 (Fig 3.3A; PACAP vs. No INJ, p=0.624; vs. vehicle, p=0.713) or just prior to M2 (Fig 3.3B; PACAP vs. No INJ, p=0.613; vs. vehicle, p=0.868). Baclofen+muscimol injections into the VMN did not alter feeding during either M1 or M2 suggesting that PACAP actions in the VMN are primarily excitatory. Every cannula placement into the VMN was confirmed at the conclusion of the study resulting in a 90% accuracy rate.

NAc microinjections

NAc injections of PACAP (Fig 3.6B for anatomy), AMPA, or baclofen+muscimol (prior to M1) had no effect on feeding behavior during M1 (Fig 3.4A; F(4,80)=0.463; p=0.763). However, intra-NAc injections of PACAP and baclofen+muscimol significantly reduced WD intake during the subsequent 15 min M2 compared to vehicle and non-injected controls (Fig 3.4A; PACAP vs No INJ, p<0.001; vs

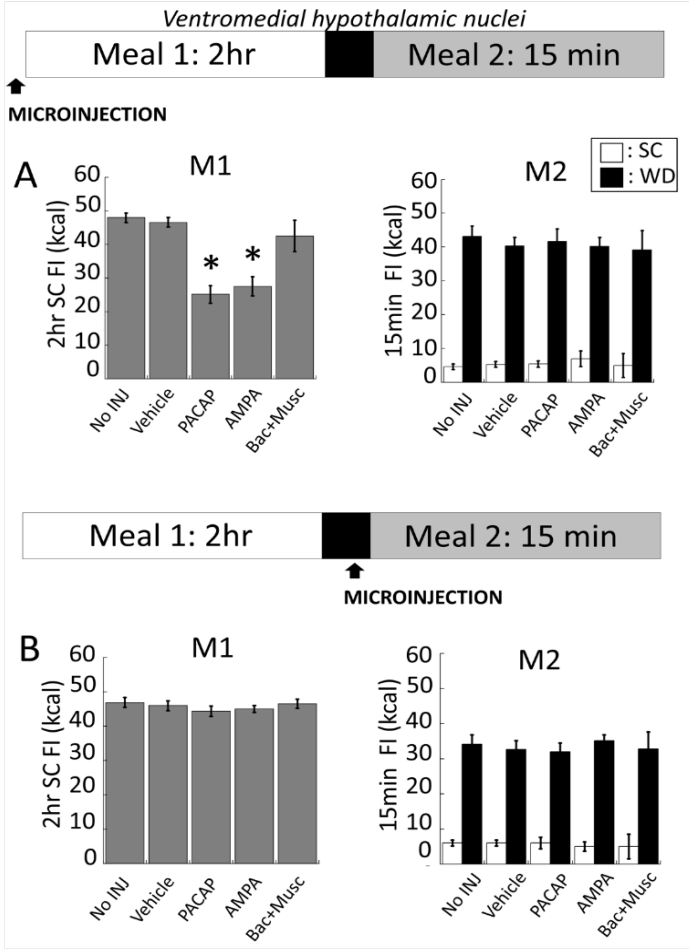


Figure 3.3 PACAP or AMPA microinjections into the hypothalamic ventromedial nuclei (VMN) suppressed homeostatic-induced feeding (meal 1; M1) without affecting palatable food consumption (meal 2; M2). **(A)** Intra-VMN PACAP or AMPA administered prior to M1 significantly suppressed homeostatic-induced feeding of standard chow (SC) compared to baclofen+muscimol, vehicle (saline) and non-injected controls, whereas there were no significant treatment difference in the consumption of SC or palatable diet (WD) during M2. **(B)** No changes in SC or WD consumption following microinjections administered just prior to M2. Data expressed as mean ± SEM. * = $p<0.05$.

vehicle, $p<0.002$; baclofen+muscimol vs No INJ or vehicle, $p<0.001$). Similarly, PACAP and baclofen+muscimol administered just prior to M2 also suppressed WD intake (Fig 3.4B; PACAP vs No INJ or vehicle, $p<0.001$; baclofen+muscimol vs No INJ or vehicle, $p<0.001$). By contrast, AMPA administration into the NAc prior to either M1 or M2 had no effect on food consumption suggesting that PACAP actions in the NAc were inhibitory. Every cannula placement into the NAc was confirmed at the conclusion of the study resulting in a 90% accuracy rate.

Slice electrophysiology

We determined whether PACAP affected action potential firing rates in VMN and NAc slices. All recordings were made in the presence of the glutamate receptor antagonist CNQX (10 μm) and the $GABA_A$ receptor blocker picrotoxin (50 μm) to block excitatory and inhibitory synaptic transmission. Cell-attached patch clamp recordings were made on VMN neurons, which displayed spontaneous action potential firing. Bath application of PACAP (100 nM) significantly increased the frequency of spontaneous action potential firing in VMN neurons (Fig 3.5A, t(6)=-4.062, *n=7,* p<0.004, Paired t-test). We next examined whether PACAP also affected action potential firing in the NAc. Since medium spiny neurons (MSNs) in NAc slices do

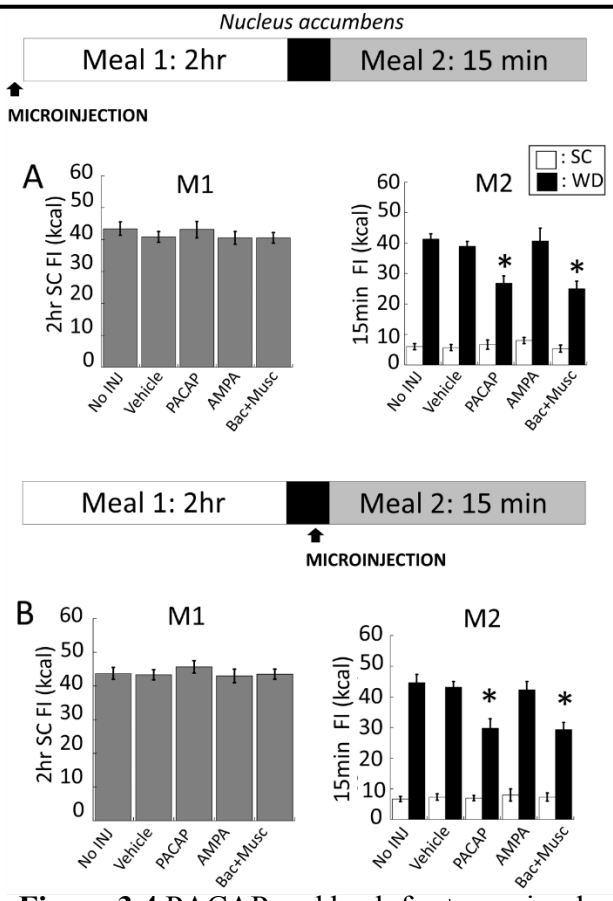


Figure 3.4 PACAP and baclofen+muscimol microinjections into the nucleus accumbens (NAc) suppressed palatable food consumption (meal 2; M2) without effecting standard chow (SC) intake (meal 1; M1). **(A)** No changes to SC intake following microinjections administered prior to M1 compared to controls. However, intra-NAc PACAP or baclofen+muscimol significantly reduced WD intake compared to AMPA, vehicle and non-injected controls during M2. **(B)** Similarly, WD intake was reduced following PACAP and baclofen+muscimol administration into the NAc just prior to M2. Data expressed as mean ± SEM. * = p<0.05.

not fire spontaneous action potentials at resting membrane potential (~-80 mV), we made whole-cell current-clamp recordings and evoked action potential firing by injecting depolarizing current steps. Bath application of PACAP (100 nM) significantly decreased the number of spikes in responses to depolarizing current injections (Fig 3.5B, 120 pA, $t(5)=4.828$, $p<0.005$; 180 pA, $t(5)= 4.620$, $p<0.006$; 240 pA, $t(5)=11.364$, $p<0.001$; 300 pA, $t(5)=5.937$, $p<0.002$, $n=6$). These effects were independent of excitatory and inhibitory synaptic inputs as these studies were conducted in the presence of both CNQX and picrotoxin. Thus, PACAP increased spontaneous action potential firing in the VMN whereas, it decreased evoked action potential firing in the NAc.

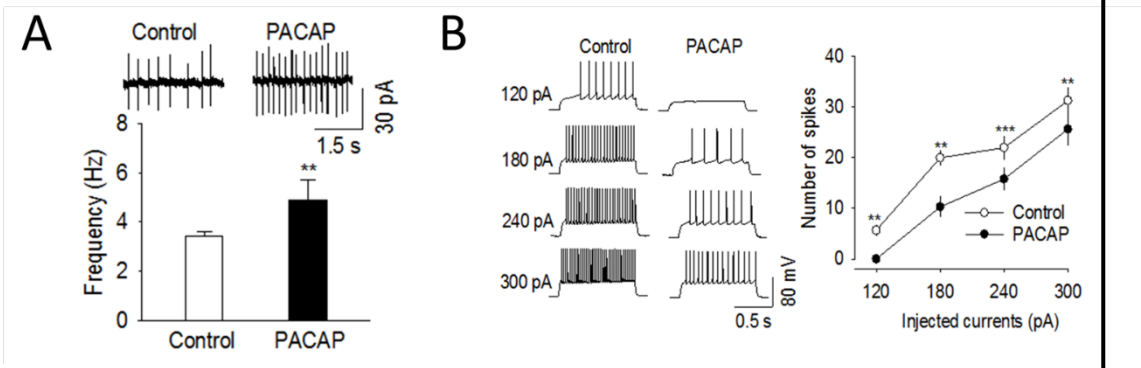


Figure 3.5 PACAP produced opposite effects on action potential firing in the VMN and NAc. **(A)** PACAP significantly increased the frequency of spontaneous action potential firing in the VMN (n=7, **p<0.01 vs control). (**B)** PACAP decreased the number of spikes in the NAc in response to depolarizing current injections (120 pA to 300 pA, n=6, **p<0.01 vs control, *** p<0.001 vs control, Paired t-test). All physiological recordings were collected in the presence of CNQX and picrotoxin.

DISCUSSION

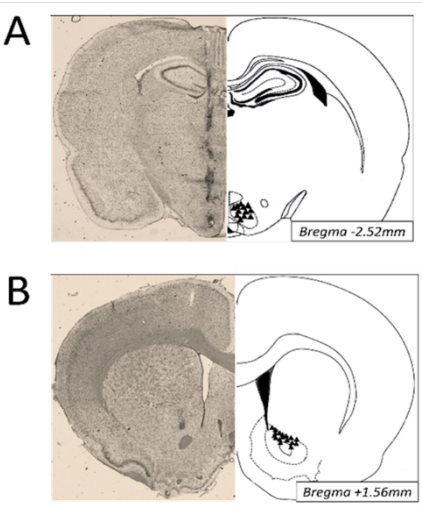


Figure 3.6 Photomicrographs of cannula placements (triangles, right) and a representative Nissl stained section (30 □m; left) of the VMN **(A)** and NAc **(B)**.

Obesity can stem from excessive or binge-like consumption of food generated by different homeostatic and hedonic-related drives, each of which may involve distinct circuitry in the brain. This study extends earlier findings revealing that PACAP administration into the hypothalamic VMN markedly suppressed feeding behavior (Resch et al., 2011; Resch et al., 2013) by determining the capacity of this novel anorexigenic peptide to regulate distinct forms of eating stemming from homeostatic and hedonic feeding drives. To do this, we developed a novel binge-eating paradigm (rapid consumption of a high volume of food within a short time period) that would better isolate distinct feeding drives. Using this paradigm, it is likely that VMN activation suppressed the consumption of standard chow (SC) in restrict-fed rats without altering palatable food intake in a satiated rat. Inhibition of the NAc produced the opposite outcome in that consumption of palatable food in a satiated rat was reduced, while SC intake was not altered. Interestingly, PACAP signaling in the VMN and NAc produced the precise changes in synaptic transmission needed to suppress each form of eating. Collectively, these data suggest that distinct feeding drives may involve at least partially non-overlapping circuitry, and that targeting PACAP signaling may be an effective strategy at reducing both homeostatic and hedonic-related feeding.

Isolation of Homeostatic- and Hedonic-related Feeding Drives

A challenge in the study of the neurobiology of obesity is that multiple feeding drives are likely *simultaneously* activated under most experimental conditions thereby, obfuscating efforts to identify the cellular or molecular basis of discrete feeding drives (Lowe & Butryn, 2007; Lowe & Levine, 2005). Many rodent models assess consumption of a highly-palatable food combined with some degree of food deprivation, thereby demonstrating the presence of multiple feeding drives even in those designed to separate distinct drives. For example, in the limited-access binge model, rodents are provided *ad lib* access to SC and limited-access to palatable foods often high in both fat and sugar (Corwin, 2004; Corwin & Hajnal, 2005; Czyzyk et al., 2010). While *ad lib* SC intake should mitigate homeostatic-driven feeding during the limited access to a highly palatable diet, rats display self-imposed deprivation evident by significantly decreased SC consumption. Thus, both homeostatic- and palatability-driven feeding drives are likely engaged during the limited access period. In the current approach, we limited the co-existence of homeostatic-driven and palatability-driven feeding drives by creating conditions in which restricted-feeding produces heightened homeostatic-driven feeding that is satiated with a low-palatable diet. It is important to note that the study of the neural mechanisms underlying feeding involving *homeostatic*- or *hedonic*-related drives requires the manipulation of these variables to understand the specific contribution of each of these drives. There are three key design aspects used to create this desired experimental condition. First, subjects were restrict fed for 2 hours; these conditions do not result in overt increases in stress hormones (Choi et al., 1998) and has previously been used by numerous others to enhance homeostatic-driven feeding (Baldo, Spencer, Sadeghian, &

Mena, 2016; Denis et al., 2015; Hagan & Moss, 1997; Wei et al., 2015). Confounding interactions stemming from malnourishment in these animals is extremely unlikely since rats with similar long-term caloric restriction paradigms (25% reduction) show improved life expectancy and health outcomes (Keenan et al., 1996; Keenan, Wallig, & Haschek, 2013). Second, SC was used as the low-palatable diet, which is illustrated in other studies to show diminished motivation for SC after exposure to a palatable diet (South, Holmes, Martire, Westbrook, & Morris, 2014). Third, the duration of the 2-HR restricted feeding of SC was sufficient to produce satiety as evident by the lack of increased consumption when the access period was lengthened to 3 or 4 hours. Hence, these conditions permit the study of the cellular or molecular basis of homeostatic-driven eating that culminates in a robust state of satiety.

In the next phase of the paradigm, palatability-driven feeding was assessed by measuring feeding in satiated rats provided additional access (15 min) to either low- or high-palatable diets. As expected, palatable food consumption significantly increased compared to the minimal consumption of SC. Remarkably, the average number of calories consumed of a highly-palatable food by satiated rats (M2) was equivalent to the number of SC calories consumed during the 2-HR M1. This marked increase in the highly palatable food is unlikely to be due to novelty or stimulus-specific satiety since a similar increase was not obtained when SC was made novel with either vanilla or almond flavorings (Fig 3.2D). Hence, these conditions likely permit the study of the cellular or molecular basis of palatable-driven feeding with limited influence from homeostatic-driven eating.

VMN Gate Homeostatic But Not Hedonic-Driven Feeding

Historically, the VMN were thought to be critical components of the brain's "satiety center" (Kennedy, 1950) and later described as the inhibitory counterpart to the lateral hypothalamus (promoting feeding) in the dual-center hypothesis for motivated behavior (Stellar, 1954). Recent studies continue to support the VMN as key sites in the regulation of energy homeostasis by demonstrating that specific genetic deletions in the VMN lead to obesity (Kim, Zhao, & Parker, 2009), altered fMRI activity is evident in the VMN after ingesting a glucose solution (Liu & Gold, 2003), and a positive correlation has been made between the degree of medial hypothalamic damage and excess weight gain (Pinkney, Wilding, Williams, & MacFarlane, 2002). However, an important outstanding question is whether the satiety signal from the VMN regulates multiple distinct feeding drives (e.g., homeostatic and hedonic-driven feeding). Using the two-meal paradigm, we found that VMN activation achieved by local AMPA injections decreased consumption of SC in restrict-fed rats but, surprisingly, it did not alter palatability-driven feeding. While more work is needed to more thoroughly characterize this effect, these findings are consistent with the conclusion that homeostatic- and palatability-driven feeding involve at least partially non-overlapping circuitry.

NAc Gates Palatability But Not Homeostatic-Driven Feeding

The NAc has been strongly implicated in a wide-range of motivated behaviors, including palatability-driven feeding (Aragona et al., 2006; Baldo & Kelley, 2007; Robbins & Everitt, 1996; Wise, 1998). However, an open question is whether NAc-related circuitry are also involved in homeostatic-driven feeding, in part because many

studies measure intake when both homeostatic- and palatability-related drives would be present. We found that local inactivation of the NAc by baclofen+muscimol reduced palatability-driven but not homeostatic-driven feeding. Our finding that GABA agonists into the NAc did not reduce homeostatic-driven consumption of SC is consistent with earlier work (Stratford & Kelley, 1997). However, we are the first to show that inhibition of the NAc decreased hedonic-driven feeding in rats that were accustomed to binge eating a palatable meal. While it is possible that regions of the NAc or ventral striatum not impacted by our manipulations may contribute to both forms of eating, our results, at a minimum, reinforce the concept that each of these feeding drives can involve unique circuitry. Illustrating this point is the evidence that GABA agonist administration in other regions of the NAc show increased feeding behavior (Basso & Kelley, 1999). Thus, discretely mapping the anatomical underpinnings of various feeding drives could provide key insight into the etiology of eating behavior underlying distinct forms of obesity. For example, individuals displaying excess eating stemming from enhanced homeostatic-driven feeding versus those that display enhanced (or the inability to suppress) palatable-driven feeding may express unique molecular and cellular pathological changes that could be targeted by more focused therapeutic intervention.

PACAP Gates Both Homeostatic- and hedonic-Driven Feeding

In the NAc, microinjections of PACAP did not alter homeostatic feeding but effectively reduced consumption of a highly palatable diet. Specifically, intra-NAc PACAP only altered consumption of high-fat, high-carb food in a satiated rat. The lack of an effect on homeostatic feeding is unlikely to be due to an insufficient dose or drug

duration given that identical parameters were used in the VMN to block homeostatic feeding and in the NAc to block palatable feeding. Interestingly, the activation of NAc efferents, all of which are GABAergic, is linked to multiple forms of motivated behavior including palatability-driven feeding, as described above (Aragona et al., 2006; Baldo & Kelley, 2007; Robbins & Everitt, 1996; Wise, 1998). Thus, our observation that PACAP in the NAc mimicked the behavioral effects of GABA agonists suggests that PACAP likely inhibited at least some of these circuits, although as discussed below the precise mechanism is unknown.

In the VMN, we found that microinjections of PACAP reduced homeostatic but not hedonic feeding. In support, PACAP microinjections into the VMN decreased consumption only when rats displayed a pronounced homeostatic drive (e.g., following a 22 HR fast). Once the animal achieved a state of satiety, PACAP microinjections into the VMN did not alter the consumption of either standard chow or a highly palatable food source. Interestingly, PACAP in the VMN mimicked the actions of AMPA microinjected into this structure. Given that previous studies have established the VMN as a satiety center of the brain in which activation of this structure reliably decreases feeding, these collective results suggest that both PACAP and AMPA excited VMN efferents involved with satiety. While our experiments did not identify the type of cell impacted by PACAP, previous studies have revealed that the majority of VMN cells are glutamatergic (Bowers, Cullinan, & Herman, 1998; Ovesjo, Gamstedt, Collin, & Meister, 2001). In support, studies have shown highly dense expression of the glutamatergic marker vGlut2 (Ziegler, Cullinan, & Herman, 2002) with minimal expression of non-glutamatergic cells.

Our finding that PACAP signaling in the VMN reduces homeostatic but not hedonic feeding extends existing work establishing the hypophagic and metabolic actions of this neuropeptide. Although PACAP signaling has been implicated in feeding behavior and body weight regulation for over 20 years (Chance, Thompson, Thomas, & Fischer, 1995; Morley et al., 1992), only recent studies have begun to delineate its regional and mechanistic details. PACAP administration into the VMN reduces *ad lib* feeding without malaise specifically through the PAC1R receptor subtype, while also increasing thermogenesis and spontaneous locomotor activity (Resch et al., 2011). Likely as a result of both the anorexia and the increased metabolic indices, PACAP in the VMN results in dramatic body weight loss even after a single acute administration (Resch et al., 2011; Resch et al., 2013). Moreover, PACAP administration in the VMN increases both POMC mRNA expression in the arcuate nuclei and fasting glucose levels further illustrating a role for PACAP in the regulation of energy balance.

Given the historical roles for the NAc in generating motivated behaviors and the VMN in suppressing feeding, it would seem that a molecule acting in each structure would need to have the remarkable capability of inhibiting the NAc while activating the VMN to regulate each form of eating. While more work needs to be done to confirm these effects for PACAP, our data are consistent with this type of region-specific regulation. In the current study, we found that bath application of PACAP to VMN slices increased action potential firing and that microinjections of PACAP and AMPA produced the same behavioral effect. In the NAc, PACAP appears to produce the opposite effect in that bath application of PACAP to NAc slices decreased evoked

potentials and microinjections of PACAP into the NAc mimicked the effects of baclofen+muscimol on feeding.

While the current results do not identify the molecular basis for PACAP mimicking GABA agonists in the NAc and AMPA in the VMN, previous work has shown that PACAP is able to increase or decrease the activity of glutamate ionotropic receptors, including NMDA (Shioda et al., 1997; Toda & Huganir, 2015; Vaudry et al., 2009). Lastly, previous work has also linked PACAP to other glutamatergic mechanisms, such as system x_c^- (Kong et al., 2016; Resch, Albano, et al., 2014) and activation of metabotropic glutamate receptors (Baker et al., 2003; Baker et al., 2002), which may display region-specific differences in expression (Gu et al., 2008). Regardless, these data show the degree to which the complexity of the glutamate network can differ across discrete brain regions yet be regulated by the same neuropeptide, potentially revealing PACAP to be a powerful regulator of caloric intake by both activating or inhibiting circuits associated with satiety (e.g., VMN) and appetitive (e.g., NAc) signals, respectively. Future studies will be needed to explore this intriguing possibility.

Collectively, these data suggest that PACAP signaling suppresses multiple feeding drives, which positions this novel anorexigenic peptide as an important target in understanding and possibly treating obesity. Toward the latter observation, identifying therapeutic targets capable of modulating multiple feeding drives may be especially important in the treatment of obesity given the widely observed propensity for tolerance to anti-obesity medications to have long-term utility (Fernstrom & Choi, 2008), an effect that could be due to compensatory changes across distinct drives. Thus, these findings

may address a fundamental barrier in treating obesity by better isolating individual feeding drives and demonstrating the potential for PACAP signaling to regulate unique forms of overeating.

CHAPTER IV

ANTAGONISM OF PAC1R-DEPENDENT SIGNALING IN THE VMN IS SUFFICENT TO BLOCK BEHAVIORAL & MOLECULAR ACTIONS OF LEPTIN.

INTRODUCTION

In Chapter III, we demonstrate that PACAP microinjected in the VMN suppresses homeostatic hunger or calorically driven feeding (Hurley et al., 2016a). In the current chapter, we demonstrate that PACAP signaling in the VMN interacts at a behavioral and molecular level with the classical adipocyte-derived hormone, leptin. The data presented in this chapter further emphasizes the point that VMN-PACAP plays a crucial role in regulating homeostatic-related feeding drives by demonstrating that the central action of leptin, which has long been thought of as a reporter of energy state (Rosenbaum, & Leibel, 2014), is dependent on endogenous PACAP in the VMN.

It has been well documented that leptin has actions both peripherally and centrally to regulate feeding behavior as well as energy metabolism (Pelleymounter, Cullen, Baker, et al., 1995; Rentsch, Levens, & Chiesi, 1995; Sidhu, Parikh, & Burman, 2000; Zhang et al., 1994). With leptin mRNA in fat cells correlated with cell size (Considine et al., 1995; Funahashi et al., 1995), excess adipose tissue in obese individuals results in chronically elevated circulating leptin levels which in time can lead to peripheral and central leptin resistance (Adeyemi & Abdulle, 2000). Moreover, this leptin-insensitivity in the regulation of feeding behavior has been suggested as one of the primary drivers leading to obesity (Berthoud, Lenard, & Shin, 2011). Taken together, understanding the mechanisms by which central leptin signaling is regulated will be necessary to pursue

effective therapeutic approaches to reverse leptin resistance and treat excess weight gain and obesity.

One of the main sites leptin signals in the brain is in the hypothalamus (Maniscalco & Rinaman, 2014), with systemic leptin administration leading to strong immune reactivity for phosphorylated signal transducer and activator of transcription-3 (P-STAT3) and c-Fos protein in the arcuate nucleus as well as the ventromedial nucleus of the hypothalamus or VMN (Elmquist et al., 1997). P-STAT3 is the phosphorylated product of the janus kinase-2 (JAK2) -STAT3 cascade (Jiang et al., 2008) that is activated when leptin binds to the leptin receptor in these two brain regions. Although the mechanism by which leptin signals in the arcuate is well characterized, less is known about the role leptin plays in the VMN (Baver et al., 2014; Beutler et al., 2017). Previous studies have demonstrated that the leptin receptor (LEPR) in the VMN is expressed on steroidogenic factor 1 (SF-1) neurons, which is significant because specific knockdown of LEPR in SF-1 neurons produces an obese phenotype in rodents (Dhillon et al., 2006; Hawke et al., 2009). Interestingly, these SF-1 neurons also express the neuropeptide pituitary adenylate cyclase-activating polypeptide or PACAP. Hawke et al., suggest that leptin and PACAP signaling interact in the VMN as they show that acute blockade of PAC1R with PACAP 6-38 administered into the third ventricle, attenuated leptin induced suppresses of food intake, body weight and changes in thermogenesis (Hawke et al., 2009).

As we have previously demonstrated that PACAP microinjected into the VMN induces hypophagia as well as other notable changes in energy expenditure (Hurley et al., 2016a; Resch et al., 2011; Resch et al., 2013; Resch, Maunze, et al., 2014), the current

study was designed to evaluate the level of interaction between PACAP and leptin specifically in the VMN. This was achieved through a number of neuropharmacological experiments as well as molecular experiments using western blotting and *in situ* hybridization. Our findings demonstrate that acute blockade of PAC1R in the VMN is enough to disrupt VMN-leptin action behaviorally and molecularly. Future studies exploring central leptin resistance should therefore, take into consideration axillary signaling mechanisms (i.e. PACAP) that appear to gate leptin signaling in the VMN.

MATERIALS & METHODS

Animals

Male Sprague-Dawley rats (Envigo; Madison, WI) weighing 275-300 grams upon arrival were housed individually in either standard tub cages or BioDAQ feeding cages (Research Diets; New Brunswick, NJ) and acclimated for one week to a climate controlled colony room under a 12:12 light-dark cycle. Animals were *ad lib* fed standard chow (Teklad rodent diet #8604; 32% protein, 54% carbohydrate, 14% fat; 3.0 kcal/g; Madison, Wisconsin) and body weights were measured daily. All animal procedures were approved by Marquette University Institutional Animal Care and Use Committee.

Surgery & microinjections

Cannulation surgery

Animals were anesthetized with a ketamine/xylazine/acepromazine (77:1.5:1.5 mg/ml/kg; i.p.) cocktail and placed in a stereotaxic apparatus. 26-gauge bilateral guide cannulae (Plastics One; Roanoke VA) were placed 3 mm dorsal to the ventromedial nuclei (VMN) of the hypothalamus and secured to the surface of the skull with acrylic resin. Stereotaxic coordinates for VMN injections were *1*) anterior/posterior, −2.5 mm from Bregma; *2*) medial/lateral, ± 0.6 mm from midline; *3*) dorsal/ventral, −6.2 mm from surface of the skull based on The Rat Brain (Paxinos & Watson, 2007). Following the conclusion of each study, animals were sacrificed and brains collected by rapid decapitation then sectioned and Nissl stained to determine cannula placement. Only those with correct placements were included in the studies.

Telemetry probe

Prior to VMN cannulation, some animals (n=15) were implanted intraperitoneally with a telemetry probe (Mini-Mitter, Sunriver, OR). Animals were allowed one week to recover from surgery before baseline body temperatures were monitored for one week prior to VMN microinjections.

Microinjections

PACAP (50 pmol/0.25µl per side; California Peptide Research, Napa, CA), PACAP 6-38 (500 pmol/0.25µl per side; Anaspec, Fremont, CA), leptin (0.025 mg/0.25µl per side; R&D Systems, Minneapolis, MN) or saline were microinjected into the hypothalamic VMN over a two minute period in gently restrained awake animals. Groups included

vehicle (n=4-11), leptin (n=6-10), PACAP (n=4-10), PACAP 6-38 (n=5), PACAP 6-38 +PACAP (n=4-10) or PACAP 6-38 + leptin (n=6-12) at the onset of the dark cycle. Afterwards an additional minute was allotted before removing injectors to prevent backflow.

In situ Hybridization

Rat brains were sectioned coronally at 12 μm and then postfixed in 4% paraformaldehyde, rinsed in 0.1 M PBS (pH 7.4), equilibrated in 0.1 M triethanolamine (pH 8.0), and acetylated in triethanolamine containing 0.25% acetic anhydride. Standard in vitro transcription methods were used to generate both sense and antisense riboprobes recognizing PAC1R, PACAP, LEPR and BDNF (Choi, Milwaukee, WI) transcripts, which were subsequently diluted in hybridization cocktail (Amresco, Solon, OH) with tRNA. Sections were hybridized overnight at 60°C with either digoxigenin (DIG) or fluorescein (FITC)-labeled riboprobes. After hybridization, slides were treated with RNase A and stringently washed in 0.3× SSC at 65°C (PAC1R/BDNF) for 30 min. Slides were incubated with an antibody against DIG or FITC conjugated to horseradish peroxidase (HRP; Roche) overnight at 4°C. Riboprobe signal was amplified using the TSA-Plus fluorophore system with either fluorescein or Cy3 (PerkinElmer; Waltham, MA). Image capture was performed using fluorescent microscopy (Axioskop-2; Zeiss, Thornwood, NY) and Axiovision image analysis software (Zeiss, Thornwood, NY).

Quantitative polymerase chain reaction.

Dissected VMN tissue was frozen in liquid nitrogen and stored at -80 °C. Total RNA was isolated from hypothalamic VMN punches by TRIzol extraction (Invitrogen-ThermoFisher) and cDNA was constructed using the Reverse Transcription System (Promega, Madison, WI). Quantitative PCR performed using a StepOne Real-Time PCR System (Applied Biosystems-ThermoFisher), and PerfeCTa SYBR Green FastMix with ROX (QuantaBio, Beverly, MA). Quantification of mRNA expression were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primers **GAPDH:** 5'-CTCCCATTCTTCCACCTTTGA-3' and 5'-ATGTAGGC CATGAGGTCCAC-3', **SOCS3**: 5' CCC CGC TTT GAC TGT GTA CT 3'and 5' AAA GGA AGG TTC CGT CGG TG 3'**, BDNF:** 5' AAA ACC ATA AGG ACG CGG ACT T 3' and 5' AAA GAG CAG AGG AGG CTC CAA 3'.

Western blotting.

VMN tissue was collected 30 min after microinjections and flash frozen in liquid nitrogen and then homogenized by hand (10 strokes) in ice-cold homogenization buffer (300 mM sucrose, 10 mM Tris-HCl, pH 7.4, 10 mM EDTA, 10 mM EGTA) containing Halt protease and phosphatase inhibitor cocktail (Pierce; Rockfork, IL), followed by 3-4 seconds of sonication. Homogenates were centrifuged at 1000 X g for 10 min at 4 °C to obtain the nuclear fractionation. Protein levels were measured using a bicinchoninic (BCA) assay (Pierce). Nuclear protein (25 μg) was run on an 8% gel by SDS-PAGE and transferred to a polyvinylidine fluoride (PVDF) membrane. Membranes were blocked in

odyssey blocking buffer (PBS; LI-COR Biosciences; Lincoln, NE) with 0.1% Tween-20 and then probed with rabbit anti-phospho-STAT3 (Tyr705) antibody (Cell Signaling; Beverly, MA) overnight at 4°C, and then an IRDye 680 CW goat anti-rabbit secondary antibody (LI-COR Biosciences; Lincoln, NE) at room temperature for 1 hour. Band intensities for Tyr705 were visualized using the Odyssey Fc Dual Mode Imaging System. Blots were stripped and re-probed for total endogenous STAT3 expression using mouse anti-STAT3 (124H6) (Cell Signaling; Beverly, MA) and IRDye 800CW donkey anti-mouse (LI-COR Biosciences; Lincoln, NE) antibodies.

Statistics.

Data are presented as means ± standard errors of the mean, and were analyzed statistically (Sigma Plot 11; SystatSoftware Inc.; San Jose, CA) by analysis of variance (with repeated measures when appropriate) or Student's t-test. Fischer LSD analysis was used for all post-hoc group comparisons. P values less than 0.05 were considered statistically significant.

RESULTS

Antagonizing VMN-PAC1R blocks leptin induced changes in feeding and energy expenditure.

VMN cannulated animals received a microinjection of either saline, leptin, PACAP 6-38 or PACAP 6-38 + leptin at the onset of the dark cycle followed by measurements of feeding behavior (Fig 4.1A; left). Repeated measures two-way ANOVA revealed significant main effects of treatment and treatment x time (Fig 4.1A; left; treatment $F(3,293)= 11.319$, $p =0.001$; treatment x time $F(18, 293)= 3.555$, $p=0.001$).

Animals acutely injected with leptin into the VMN ate significantly less standard chow as compared to vehicle injected animals (Fig 4.1A; left; saline vs. leptin; p= 0.001). Interestingly, pretreating animals with a VMN microinjection of PACAP 6-38 prior to leptin injection, displayed no significant suppression of feeding behavior as compared to the control (Fig 4.1A; left; saline vs. PACAP 6-38 + leptin; p= 0.792). Blocking PAC1R-dependent activity in the VMN alone produced no significant effect on feeding behavior (Fig 4.1A; left; saline vs. PACAP 6-38; p= 0.708). Analysis of 24-hour body weight post microinjection with one-way ANOVA resulted in a significant main effect of treatment (Fig 4.1A; right; treatment $F(3,41)= 31.002$; p= 0.001). With leptin treatment inducing profound hypophagia, leptin injected animals also lost a significant amount of weight compared to control treated animals (Fig 4.1A; right; saline vs. leptin; p= 0.001). Animals pretreated with PACAP 6-38 prior to leptin microinjection displayed similar weight gain to saline injected animals (Fig 4.1A; right; saline vs. PACAP 6-38 + leptin; p= 0.792).

In addition to investigating VMN leptin-PACAP regulation on feeding behavior, in a separate study, we also analyzed regulation of thermogenesis. Analysis of core body temperature post-microinjection of either saline, leptin or PACAP 6-38 + leptin produced a significant effect of treatment over time using a two-way repeated measures ANOVA (Fig 4.1B; treatment x time $F(38,279)=1.572$, p= 0.025). Post-hoc analysis revealed animals injected with leptin alone displayed a prolonged significant increase in core body temperature that was still significantly elevated 20 hours post injection as compared to vehicle (Fig 4.1B; vehicle vs. leptin, p=0.002). Interestingly, antagonizing PAC1R with PACAP 6-38 prior to administration of leptin resulted in no significant difference in core

body temperature as compared to vehicle injected animals at this same time point (Fig 4.1B; PACAP 6-38 + leptin vs. leptin, p= 0.385). Increases in body temperature subsided by 30 hours post injection. Taken together, our data suggests endogenous PAC1R

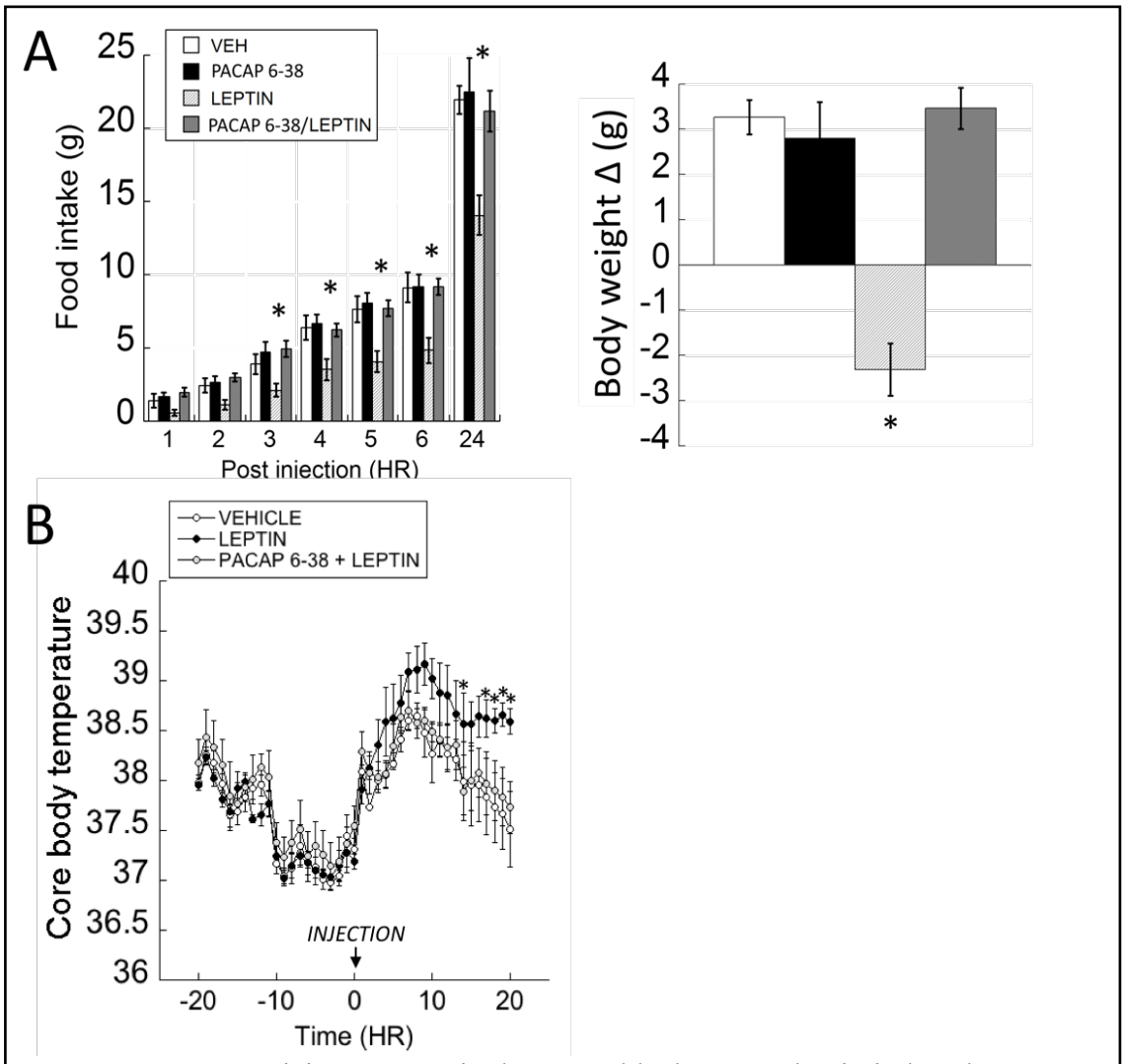


Figure 4.1 Antagonizing PAC1R in the VMN blocks VMN-leptin induced hypophagia, weight loss and thermogenesis. **(A, left)** Bilateral VMN-leptin (.025ug/.25µl/side) significantly suppressed feeding behavior and this suppression was blocked with pretreatment with PACAP 6-38 (500 pmol/.25µl/side). **(A, right)** A similar pattern of data was observed in body weight change 24HRs post microinjection. **(B)** Leptin bilaterally microinjected into the VMN stimulated thermogenesis significantly up to 20 hours post inject compared to vehicle and PACAP 6-38 pretreated animals. Data is presented ± SEM. * = $P < 0.05$

dependent activity in the VMN is critical for leptin-induced changes on food intake, body weight and thermogenesis.

PAC1R mRNA expressing cells in the VMN also express PACAP, BDNF and LEPR.

As Hawke and colleagues demonstrated that SF-1 neurons of the VMN also express PACAP (Hawke et al., 2009), we determined whether these SF-1/PACAP neurons also expressed the PACAP receptor PAC1R. Using double *in situ* hybridization targeting mRNA transcripts for PACAP and PAC1R, we found both signals to be expressed in the same cells (Fig 4.2A). Additionally, we demonstrated that PAC1R was

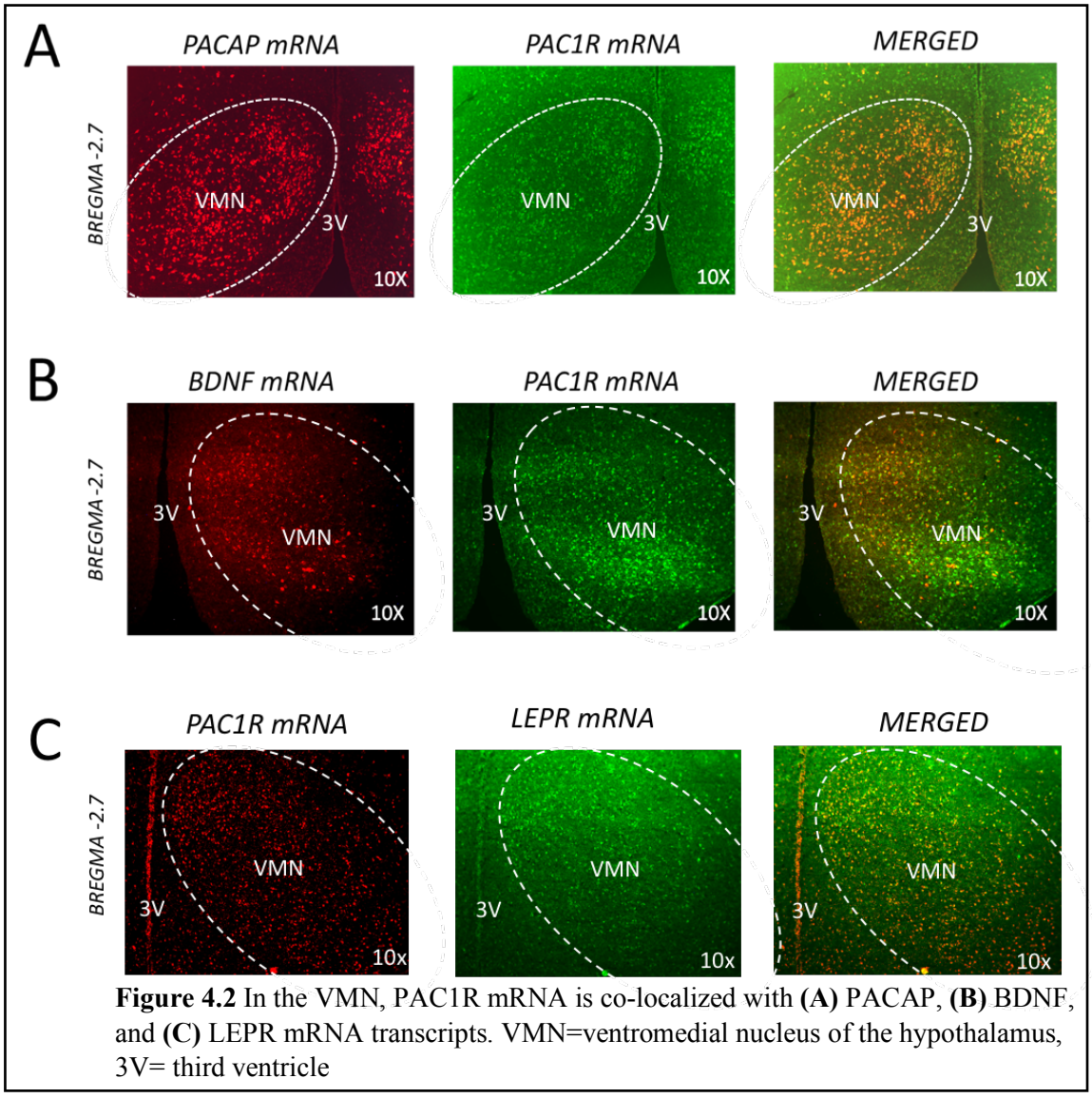


Figure 4.2 In the VMN, PAC1R mRNA is co-localized with **(A)** PACAP, **(B)** BDNF, and **(C)** LEPR mRNA transcripts. VMN=ventromedial nucleus of the hypothalamus, 3V= third ventricle

also co-localized with brain derived neurotrophic factor (BDNF; Fig 4.2B) and the leptin receptor (LEPR; Fig 4.2C) in the VMN.

Evidence for PACAP-leptin interaction at the molecular level in the VMN.

It has been well documented that activation of the leptin receptor drives the JAK2-STAT3 intracellular signaling cascade (Jiang et al., 2008), which in brief, increases phosphorylation of P-STAT3 leading to the transcription of a number of genes, including suppressor of cytokine signaling-3 (SOCS3), which effectively blocks the phosphorylation of JAK2 and subsequently inhibits the signaling cascade. Importantly, previous studies have demonstrated that central and systemic leptin increases P-STAT3 immunoreactivity in the VMN (Ladyman, Fieldwick, & Grattan, 2012). In the current study, we examined whether PACAP microinjection in the VMN would stimulate STAT3 phosphorylation and gene transcription similar to those observed following leptin administration. PACAP stimulated phosphorylation of STAT3 in the VMN, which was dependent on PAC1R activation since PACAP 6-38 blocked the increase in STAT3 phosphorylation (Fig 4.3A; treatment $F(2,11)=7.733$, $p=0.11$; vehicle vs. PACAP $p=0.029$; PACAP 6-38 + PACAP vs PACAP $p= 0.014$; vehicle vs. PACAP 6-38 + PACAP $p=0.674$). In addition, leptin administration into the VMN increases mRNA levels of BDNF as well as suppressor of cytokine signaling-3 (SOCS3) thus, we examined whether PACAP would produce similar changes in gene expression and whether the PACAP-induced and/or leptin-induced gene expression could be blocked through PAC1R (Fig 4.3B). VMN cannulated animals were injected with either vehicle, PACAP 6-38, PACAP, PACAP 6-38 + PACAP, leptin or PACAP 6-38 + leptin and

sacrificed 3 hours post microinjected and VMN tissue was collected for qPCR analysis of BDNF and SOCS3 mRNA. Both leptin and PACAP treated groups displayed similar significant increases in BDNF mRNA expression (Fig 4.3B left; treatment $F(5, 46)= 6.800$, $p< 0.001$; vehicle vs. PACAP $p<0.001$; vehicle vs. leptin $p<0.001$). However, pretreating with PACAP 6-38 prior to receiving a VMN injection blocked the effects of PACAP and leptin on BDNF levels (Fig 4.3B left; vehicle vs. PACAP 6-38 + PACAP, $p=0.950$; vehicle vs. PACAP 6-38 + Leptin, $p=0.405$). Like BDNF, a one-way ANOVA of SOCS3 mRNA expression revealed a significant effect of treatment (Fig 4.3B right; treatment $F(5,45)= 2.901$, $p=0.025$). Post-hoc analysis of PACAP or leptin treated animals showed significantly higher mRNA levels between PACAP and PACAP+PACAP 6-38 groups ($p=0.006$) and leptin and leptin+PACAP 6-38 groups ($p=0.050$) (Fig 4.3B right). Antagonizing PAC1R prior to leptin or PACAP treatment produced similar SOCS3 mRNA levels to the vehicle treated group (Fig 4.3B right; vehicle vs. PACAP 6-38 + PACAP, $p=0.376$; vehicle vs. PACAP 6-38 + leptin, $p=0.441$).

DISCUSSION

A number of preclinical rodent studies demonstrate that exogenous application of PACAP or leptin centrally or directly into the ventromedial nucleus of the hypothalamus (VMN) produces similar behavioral effects. Specifically, they both induce long lasting hypophagia, increased thermogenesis and ultimately enhanced weight loss (Jacob et al., 1997; Morley et al., 1992; Rentsch et al., 1995; Resch et al., 2011). The similarity in behavioral outcomes suggest that these two neuropeptides could use overlapping pathways to regulate feeding behavior and metabolism. Previously, intraventricular

administration of a PAC1R antagonist has been shown to block leptin induced changes in food intake, body weight and thermogenesis (Hawke et al., 2009). With numerous brain regions showing both PAC1 and leptin receptor expression (Hashimoto et al., 1996; Scott et al., 2009), it is difficult to identify the specific brain region in which intracerebroventricular (ICV) administration of PACAP and leptin would have regulated these behavioral effects. The current study addresses this gap by evaluating PACAP and leptin interactions specifically in the VMN.

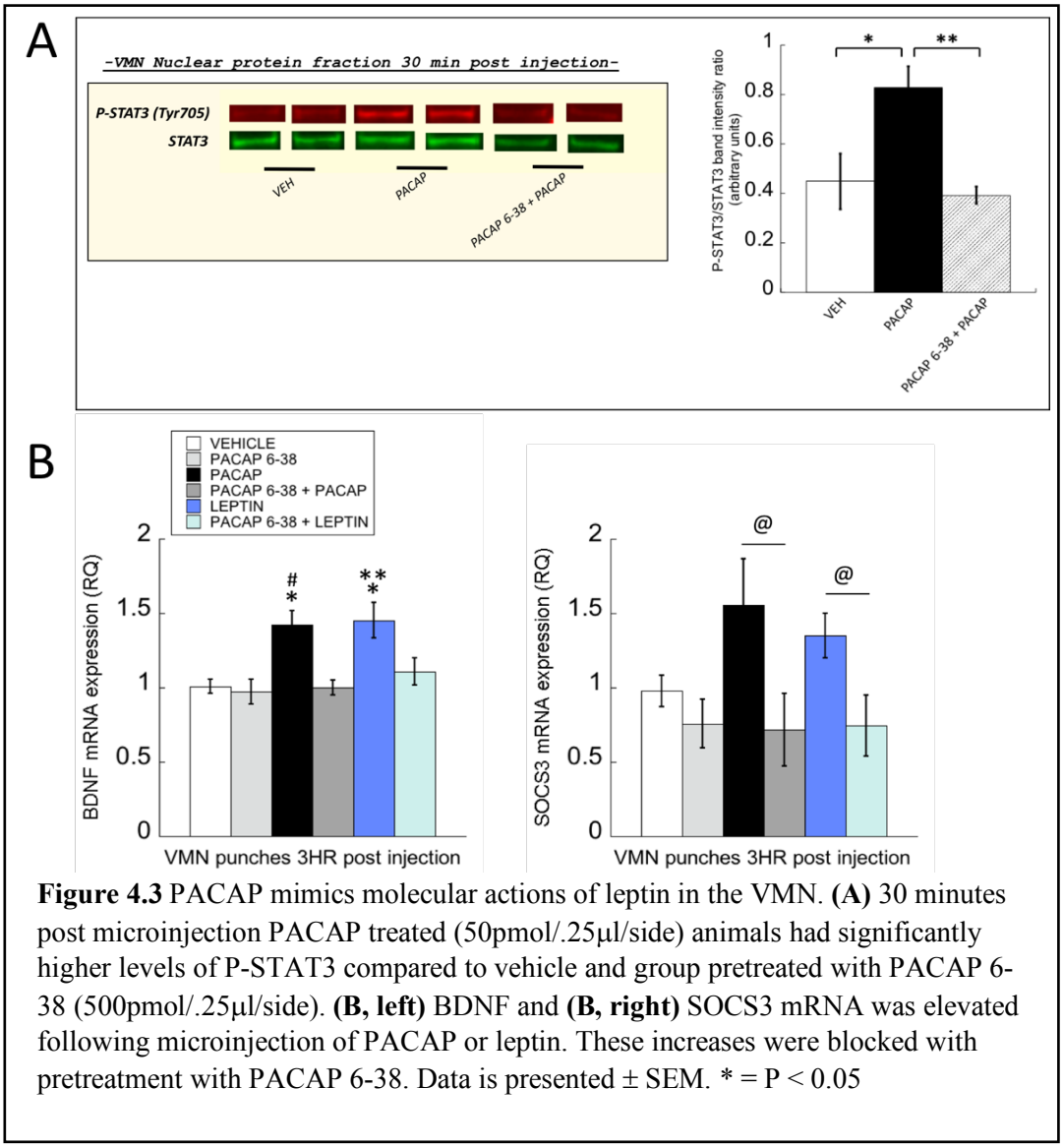


Figure 4.3 PACAP mimics molecular actions of leptin in the VMN. **(A)** 30 minutes post microinjection PACAP treated (50pmol/.25µl/side) animals had significantly higher levels of P-STAT3 compared to vehicle and group pretreated with PACAP 6-38 (500pmol/.25µl/side). **(B, left)** BDNF and **(B, right)** SOCS3 mRNA was elevated following microinjection of PACAP or leptin. These increases were blocked with pretreatment with PACAP 6-38. Data is presented ± SEM. * = $P < 0.05$

The current study's findings are consistent with previous behavioral results following ICV administration of the PAC1R antagonist, PACAP 6-38, and leptin (Hawke et al., 2009). We confirmed that leptin alone injected into the VMN suppressed food intake, induce weight loss as well as stimulated thermogenesis as has been previously reported (Choi & Dallman, 1999; Choi et al., 1999; Dhillon et al., 2006). However, prior PACAP receptor antagonism in the VMN by PACAP 6-38 prevented leptin-induced changes in food intake and metabolism (Fig 4.1). In the VMN, PAC1R mRNA is co-localized with PACAP, BDNF and LEPR mRNA transcripts (Fig 4.2). Since central administration of PACAP and leptin produce similar behavioral and metabolic effects, we investigated whether PACAP in the VMN would also produce similar effects to ICV leptin on STAT3 phosphorylation (Hubschle et al., 2001). In line with the behavioral similarities between PACAP and leptin, PACAP robustly increased nuclear P-STAT3 protein levels in the VMN, which could be blocked by PAC1R antagonism (Fig 4.3A). As a result of increased P-STAT3, exogenous leptin injections into VMN subsequently lead to increases in brain-derived neurotrophic factor (BDNF) and suppressor of cytokine signaling 3 (SOCS3) mRNA. PACAP administration alone produced similar activation of both BDNF and SOCS3 in the VMN through the PAC1R since the PAC1R selective antagonist blocked increases in both mRNA levels (Fig 4.3B).

One possible explanation for PACAP-leptin interaction is that PAC1R dependent activation of a second messenger cascade in the hypothalamic VMN functions to gate leptin receptor function, and without PAC1R activity leptin is incapable of inducing behavioral or molecular effects. Previously, we have demonstrated that PACAP induced

hypophagia could be blocked by pretreatment with PP1, a Src kinase inhibitor (Resch, Maunze, et al., 2014). This key finding illustrates that PACAP's potent action in the VMN on feeding behavior is dependent, in part, on Src kinase activity and suggests a possible intersection point leading to the phosphorylation of STAT3, *independent* of JAK2 activation (Jiang et al., 2008). This is further supported by *in vitro* data demonstrating Src kinase activation enhances leptin induced STAT3 phosphorylation (Jiang et al., 2008). Future studies will need to be conducted to determine if PACAP or leptin induced P-STAT3 and associated mRNA changes are dependent on the Src kinase activity.

Our findings also demonstrate that BDNF in the VMN could be positioned as a common downstream effector through which PACAP and leptin regulate energy expenditure. VMN BDNF levels are extremely sensitive to energy state and VMN-BDNF function is required for leptin induced hypophagia (Conner, Lauterborn, Yan, Gall, & Varon, 1997; Liao et al., 2012; Xu et al., 2003). Similar to leptin and PACAP, systemic and direct VMN administration of BDNF strongly induces hypophagia (Pelleymounter, Cullen, & Wellman, 1995; Wang, Bomberg, Billington, Levine, & Kotz, 2010), whereas knockdown of BDNF globally or specifically in the VMN produces hyperphagia and obesity (Lyons et al., 1999; Mou et al., 2015; Unger, Calderon, Bradley, Sena-Esteves, & Rios, 2007). Future studies will need to assess if PACAP-induced behavioral and molecular changes are similarly dependent on BDNF signaling.

In conclusion, our findings confirm that central PACAP and leptin interaction is observed at the level of the hypothalamic VMN. Leptin resistance is often thought of as a problem with leptin signaling but our findings suggest that disruption to an auxillary

signaling cascade involving a different neuropeptide (i.e. PACAP) may also be essential for VMN-leptin behavioral and molecular actions. Continued interrogation of the mechanisms by which leptin signals may lead to novel insights on central leptin resistance, and hopefully novel therapeutic approaches to combat obesity.

CHAPTER V

PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE (PACAP) ATTENUATES HEDONIC DRIVE IN THE ACCUMBENS.

INTRODUCTION

Unfortunately, the development of effective therapeutic approaches to treat obesity has remained stagnant due, in part, to the overwhelming complexity of an energy homeostasis system that regulates vastly diverse brain processes ranging from fat metabolism to hedonic drive (Ahima & Antwi; Kelley, 2004; Matafome & Seica, 2017; Saper, Chou, & Elmquist, 2002). In the previous chapter, we provide evidence that a component of the PACAP system regulates homeostatic hunger, in part, through behavioral and molecular interactions with the homeostatic hormone leptin in the VMN. In the current chapter we demonstrate that PACAP microinjected into the nucleus accumbens (Fig 5.2), and not the VMN (Fig 5.1), regulates the *hedonic perception* of a highly palatable 1% sucrose solution (Hurley et al., *in revision*). This demonstrates that PACAP signaling regulates a *different aspect* of motivated feeding behavior that is tied to hedonics rather than homeostasis.

There are clear psychological distinctions between 'wanting' and 'liking'. First, 'wanting' or incentive salience (Berridge & Robinson, 1998; Everitt & Robbins, 2005; Salamone & Correa, 2002), is the phenomena where cues (i.e. sight, smell, hearing, taste, etc.), which are associated with specific rewards, can trigger activity in the brain's reward circuitry in way that increases craving or *wanting* of a particular reward associated with a cue. In rodents 'wanting' is studied through the use of progressive ratio paradigms, which measures the amount of increasing effort an animal would willingly engage in for a

particular reward (Berthoud, Zheng, & Shin, 2012). On the other hand, 'liking' is specifically the hedonic impact of a reward, which can be interpreted as how much the animal enjoyed a reward (Berridge, 2009). It is important to note that 'wanting' and 'liking' work in tandem to regulate motivated behavior but are different in that wanting is the act of seeking or craving a reward, whereas 'liking' is purely the rewarding experience of ingesting the reward. For example, the hedonic impact of a reward will, in part, determine how much 'wanting' behavior is displayed when cues associated with the reward is presented (Reilly, 1999; Yoneda et al., 2007). To distinguish hedonic impact, 'liking' and 'disliking' can be experimentally quantified using the well-accepted taste reactivity behavioral paradigm, first outlined by Grill and Norgren (Grill & Norgren, 1978). In rodents, experimenters can deliver a sweet or aversive tastant through an intraoral catheter, directly or *involuntarily* into the animal's mouth. The method of tastant delivery is important because there is no aspect of 'wanting' or goal-directed action when using this experimental design. In brief, infusion of a solution into a rodent's mouth will produce a diverse set of orofacial responses involving the mouth, tongue and paws indicating whether the animal likes or dislikes a particular stimulus (Berridge, 2000). Another advantage of using this rodent model is that these orofacial reflexes are also observed in humans as well as nonhuman primates, which speaks to the conserved evolutionary importance of these hedonic reflexes (Steiner, Glaser, Hawilo, & Berridge, 2001).

We hypothesize that PACAP microinjected into the nucleus accumbens (NAc) regulates hedonic hunger (Hurley et al., 2016a) by decreasing the hedonic perception of the rewarding stimuli. The current studies test this hypothesis by comparing the effects of

PACAP microinjected into the VMN or NAc on hedonic processing using a taste reactivity paradigm. Our results not only support a selective role for PACAP signaling in the NAc to alter hedonic perception but that there is also a rostral-caudal gradient for the efficacy of PACAP within the intra-NAc core.

In addition to examining the impact of PACAP on the hedonic encoding of a rewarding stimuli, it was also pertinent to determine potential afferent projections responsible for the effect of PACAP on palatability in the nucleus accumbens. Through the combined use of retrograde tracing (cholera toxin subunit-b) and *in situ* hybridization, we identified PACAP mRNA expressing cells in the prelimbic cortex (PLC) projecting to the nucleus accumbens core (Fig 5.3). This is significant because previous studies have demonstrated stimulation of the PLC in rats decreases binge eating (Sarica et al., 2018), while pharmacological inactivation of the PLC specifically increases binge eating in rodents (Corwin et al., 2016). In line with these findings, a study performing transcranial stimulation of the prefrontal cortices in obese individuals led to a significant reduction in food intake and increases in weight loss (Gluck et al., 2015). Finally, as previously stated, binge eating rodents display diminished performance in tasks designed to test prefrontal functionality, which could suggest prefrontal dysregulation or hypofrontality may contribute to the development of binge eating behavior (Chawla et al., 2017). The use of *in situ* hybridization combined with retrograde tracing to identify afferents also confirmed the presence of the PAC1 receptor in the accumbens (5.4). Additional studies will need to examine whether other classic accumbens circuits (amygdala, hippocampus, midbrain) also project PACAP containing afferents.

The nucleus accumbens is a critical center for the regulation of reward related behaviors because it is densely innervated by dopaminergic afferents arising from the ventral tegmental area (Wise & Bozarth, 1987). The primary projection neurons in the nucleus accumbens are GABAergic medium spiny neurons (MSN), which can be broadly divided into two cell types: cells expressing D1-like receptors or cells expressing D2-like receptors. Only 5% of the MSN projections express D1-like and D2-like receptors (Bertran-Gonzalez et al., 2008). When an animal experiences a sweet tastant or ingests a high fat or sugar solution dopamine levels significantly increase in the nucleus accumbens, which leads to the subsequent activation of the two different subtypes of dopamine receptors (Avena et al., 2006; Hajnal, Smith, & Norgren, 2004; Liang, Hajnal, & Norgren, 2006). With D1-like receptors coupled to Gs and D2 like-receptors coupled to Gi, dopamine release into the accumbens following a reward (i.e. palatable food) increases excitability in D1 containing cells while decreasing the excitability of D2 expressing cells (Missale, Nash, Robinson, Jaber, & Caron, 1998; Neve, Seamans, & Trantham-Davidson, 2004). Thus conclusions have been drawn that D1-like cells are responsible for encoding reward and drive approach behavior, whereas D2-like cells are responsible for encoding aversion and avoidance behavior (Soares-Cunha, Coimbra, Sousa, & Rodrigues, 2016). In support of this, *in vivo* electrophysiology recordings in the posterior nucleus accumbens core of a rodent ingesting a sucrose solution results in decreased firing in some cells, whereas other populations of cells increase their firing (Krause, German, Taha, & Fields, 2010). Interestingly, a separate study demonstrated that rodents ingesting a high glucose or high fat solution produced immediate early gene expression in the core of the nucleus accumbens but not the shell (Dela Cruz et al., 2015).

Pharmacological studies have demonstrated that blockade of either dopamine receptor cell populations in the core or shell of the nucleus accumbens had no effect on homeostatic hunger-based feeding (Baldo, Sadeghian, Basso, & Kelley, 2002). Although more sophisticated chemogenetic approaches demonstrated that activation of D1 neurons in the accumbens increased feeding behavior, whereas inhibition of these neurons decreased feeding behavior (Zhu et al., 2016). Excessive stimulus-induced dopamine release can lead to changes by which the nucleus accumbens senses dopamine. Studies have found D2 mRNA is decreased and D1 mRNA is increased in rodent models of binge eating disorder (Colantuoni et al., 2001; Johnson & Kenny, 2010). Interestingly, obese individuals with BED have increased dopamine release in the striatum following food stimulus compared to obese individuals without BED (Wang et al., 2011). This may explain why obese individuals self-report higher levels of pleasure and display stronger reinforcement for specifically high fat and/or high sugar foods, compared to lean controls (Rissanen et al., 2002; Saelens & Epstein, 1996). As PACAP inhibits MSN's as well as suppresses palatable food intake when injected into the nucleus accumbens core (Hurley et al., 2016a), we hypothesize that PACAP dampens palatable food-induced activation of D1 cells and subsequently decrease the hedonic impact of the rewarding stimulus and drive to pursue palatable rewards. To test this, we entrained nucleus accumbens cannulated animals to the two-meal binge paradigm and pretreated them with a D1 agonist immediately prior to a PACAP microinjection (Fig 5.5). Our findings presented in this chapter suggest that in the nucleus accumbens PACAP and dopamine do, indeed, interact on a behavioral level.

Besides dopamine, findings in this chapter suggesting PACAP in the nucleus accumbens also functions through the glial cystine-glutamate antiporter, system xc- (Fig 5.6). System xc- is a heterodimer that is attached by a disulfide bond (Bridges, Natale, & Patel, 2012) and consists of a 4F2 heavy chain and the xCT light chain, which is the unique functional component of the transporter. System xc- exchanges extracellular cystine for intracellular glutamate at a 1:1 ratio (Sato et al., 1999). Recently, Kong and colleagues demonstrated that PACAP *in vitro* increases cysteine uptake only in cortical glial cells and not pure neuronal cultures and that this effect could be blocked by application of the system xc- antagonist, sulfasalazine, indicating glia are the primary cell type impacted by PACAP facilitated system xc- activity (Kong et al., 2016). PACAP-induced activity of system xc- on astrocytes leads to increases in non-vesicular glutamate release through system xc-. This link between system xc- and PACAP is exciting because system xc- activity is heavily implicated in the generation of compulsive behavior in drug addiction models (Baker et al., 2003). To determine if system xc- was necessary for PACAP-induced hypophagia in the nucleus accumbens core, we entrained nucleus accumbens cannulated rats to the two-meal paradigm that had a mutation in the gene responsible for system xc- rendering animals without a functional cystine-glutamate anti-porter. Our results suggest systemic xc- is necessary for normal PACAP mediated regulation of palatable food intake in the nucleus accumbens.

MATERIALS & METHODS

Animals.

Male Sprague-Dawley rats (Envigo; Indianapolis, IN) weighing between 250-275 grams or system xc- mutated rats (see details below), were housed individually in standard tub cages or wire bottom hanging cages in a climate-controlled colony room under a 12:12 light/dark cycle. Animals had *ad lib* access to standard chow (Harlan Diet #8604) throughout the duration of the study unless placed in the two-meal binge paradigm. Additionally, food intake and body weight were recorded daily. All animal procedures were approved by the Marquette University Institutional Animal Care and Use Committee.

System xc- mutated rats.

The laboratory of Dr. Aron Guerts from the Medical College of Wisconsin used Zinc-finger nucleases (ZFNs) technology to target the second exon of the Slc7a11 gene in rats (TGCTAGCTTTTGTTCgagtcTGGGTGGAACTGCTG). In brief, ZFNs were injected into the pronucleus of Sprague-Dawley embryos and ultimately a mutant animal was identified with a single-step, whole animal disruption of 39 consecutive base pairs in the Slc7a11 gene confirmed by sanger sequencing.

Surgery.

Animals were anesthetized with ketamine/xylazine/acepromazine (77:1.5:1.5 mg/ml/kg; i.p.) and implanted with an intraoral catheter for tastant delivery, as described previously (Wheeler et al., 2008). 26-gauge guide cannulae (Plastics One; Roanoke, VA) were stereotaxically placed 2 mm above the nucleus accumbens core (NAc; anterior/posterior +1.2 mm from bregma; medial/lateral +2.2 mm from midline; dorsal/ventral -4.8 mm from the surface of the skull, at 6° angle) or 3 mm above the ventromedial hypothalamic nucleus (VMN; anterior/posterior, −2.5 mm from bregma; medial/lateral, ± 0.6 mm from midline; dorsal/ventral, −6.2 mm from surface of the skull) and then secured to the surface of the skull using acrylic dental cement (Paxinos & Watson, 2007). Animals were given one week to recover prior to experimentation. Following the conclusion of the study, brains were collected and analyzed for cannula placement using Nissl staining, only those with correct placements were included in the studies.

Cholera toxin subunit B microinjections.

Animals (n=4) were anesthetized as described above and placed in a stereotaxic apparatus. Experimenters then injected 0.5ul of a sterile 10% cholera toxin subunit B solution unilaterally into the nucleus accumbens core (AP: +1.5; ML: +2.2; DV:-6.8) over a ten minute period. One week after surgery, animals were euthanized, and brains were collected for *in situ* hybridization.

Microinjections.

Pituitary adenylate cyclase-activating polypeptide (PACAP; n =4-11; 50 pmol/0.25µl/side in the VMN and 100 pmol/0.5µl/side in the NAc California Peptide Research; Napa, CA), SKF 81297 (1.2ug/0.5µl/side; n=5; Tocris Bioscience; Minneapolis, MN), Baclofen + Muscimol (Bac + Musc; n=4; 106.8/5.7 ng/side; Tocris Bioscience; Minneapolis, MN) or saline were microinjected over a two-minute period in gently restrained awake animals followed by an additional minute to prevent backflow.

Taste Reactivity.

Design

Following one week to recover from surgery, animals were habituated to the taste reactivity chamber for 3 consecutive days for 30 minutes/day. On test day, a 50 mL syringe containing a 1% sucrose solution was connected to the animal's intraoral catheter. The syringe was then locked into a syringe pump, which infused the sucrose solution at a rate of 1mL/min over a 1 minute trial. Each trial began 15 min following the intracranial microinfusion, and appetitive and aversive orofacial responses were video recorded using a camera fixed beneath the Plexiglas floor of the testing chamber. Animals were given a 48-hour washout period before receiving a second, counterbalanced, microinjection of either vehicle or PACAP.

Video scoring

Digital video files of each session were analyzed frame-by-frame over the one-minute test trial. Scored in 10 second bins, occurrences of lateral tongue protrusions and paw licks were counted as appetitive taste reactivity while gapes and paw flails were

evidence of aversive taste reactivity, as described in previous reports (Wheeler et al., 2015). After the six 10 second bins are scored frame-by-frame, an animal is assigned an "appetitive responses" and an "aversive responses" score which are the cumulative number of appetitive and aversive responses made by that animal over the one minute trial. The figures in this chapter reflect the cumulative appetitive and aversive scores.

Calculating PACAP-induced change in aversive response

The y-axis on figure 5.2C is reporting within-subject, PACAP-induced changes in aversive responses, normalized to vehicle trial aversive responses. To calculate this, we used the following equation:

$$\left(\begin{matrix} total\ aversive\ respones\ on\ PACAP\ trial \div \\ total\ aversive\ responses\ on\ vehicle\ trial \end{matrix}\right) - 1 = PACAP\ induced\ \Delta\ in\ aversive\ response$$

A result greater than zero indicates that PACAP increased aversive responses, whereas a result less than zero would indicate PACAP decreased aversive responses to the sucrose solution.

Two meal binge eating design

Rats were entrained to consume their daily SC intake in a 2-hour period after the onset of the dark phase (Meal 1; M1). After establishing consistent feeding patterns and weight gain (40-50 kcal/2hr; body weight gain 2-3 grams/day), animals were offered a short 15 min meal (Meal 2; M2) of either SC or WD approximately 30 minutes following M1 for 7 days before experimentation. Food intake and body weight measurements were

recorded in an additional group of rats that were *ad lib* fed either SC or WD and functioned as additional control groups.

In situ hybridization

Rat brains were sectioned coronally at 12 μm thickness and then postfixed in 4% paraformaldehyde, rinsed in 0.1 M PBS (pH 7.4), equilibrated in 0.1 M triethanolamine (pH 8.0), and acetylated in triethanolamine containing 0.25% acetic anhydride. Standard in vitro transcription methods were used to generate both sense and antisense riboprobes recognizing PAC1R, PACAP, LEPR and BDNF (Choi, Milwaukee, WI) transcripts, which were subsequently diluted in hybridization cocktail (Amresco, Solon, OH) with tRNA. Sections were hybridized overnight at 60°C with either digoxigenin (DIG) or fluorescein (FITC)-labeled riboprobes. After hybridization, slides were treated with RNase A and stringently washed in 0.3× SSC at 65°C (PAC1R/BDNF) for 30 min. Slides were incubated with an antibody against DIG or FITC conjugated to horseradish peroxidase (HRP; Roche) overnight at 4°C. Riboprobe signal was amplified using the TSA-Plus fluorophore system with either fluorescein or Cy3 (PerkinElmer; Waltham, MA). Image capture was performed using fluorescent microscopy (Axioskop-2; Zeiss, Thornwood, NY) and Axiovision image analysis software (Zeiss, Thornwood, NY).

Statistics.

Using paired t-tests, group differences between hedonic responses to sucrose pre-and post-intracranial microinfusions were compared using Sigma Plot 11 software (Systat

Software Inc.; San Jose, CA). Additionally, a one-way analysis of variance (ANOVA) was used in the experiment involving the two-meal paradigm. Fisher-LSD post hoc test was used when a main effect of significance was found. P values < 0.05 were considered statistically significant.

RESULTS

Ventromedial nucleus of the hypothalamus (VMN)

The data are presented as cumulative appetitive (lateral tongue protrusion + paw licks) and aversive (gapes + paw flails) responses to a sucrose solution following either vehicle or PACAP microinjections into the ventromedial nuclei of the hypothalamus (VMN). Within-subject results demonstrated that PACAP microinjected into the VMN had no effect on the expression of either appetitive (Fig 5.1A; *paired t-test*; t= -0.0625; DF= 10 p=0.951) or aversive responses to the sucrose solution compared to the vehicle trial (Fig 5.1B; *paired t-test*; t= -1.203; DF=10; p=0.257).

Nucleus accumbens core (NAc)

A separate group of animals were implanted with cannula targeting the NAc as well as intraoral catheters and tested in an identical manner. Rats received either PACAP or vehicle 15 minutes prior to taste reactivity testing as described above. Interestingly, PACAP microinjected into the NAc significantly suppressed appetitive responses (lateral tongue protrusions and paw licks combined) to sucrose (Fig 5.2A; *paired t*-test; t= 3.8; DF=9; p=0.004). Although PACAP did not significantly alter aversive responses to sucrose (gapes and paw flails combined), compared to vehicle (Fig 5.2B; *paired t-test*; t= -1.163; DF=9; p=0.275), the variance in the effect of PACAP appeared to be related to

the rostral-caudal placement of the microinjection. A Pearson correlation revealed a strong rostral-caudal influence of PACAP on the change in expression of aversive taste reactivity between vehicle and drug treated groups ($r = -0.64$, $n = 10$, $p = 0.04$; Fig 5.2C). Specifically, PACAP microinjected in the caudal NAc (Fig 5.2 bottom; bregma +1.68 to +1.32) increased aversive responses to sucrose, while PACAP microinjected into the rostral NAc (Fig 5.2 top; bregma +2.02 to +1.92) decreased aversive responses.

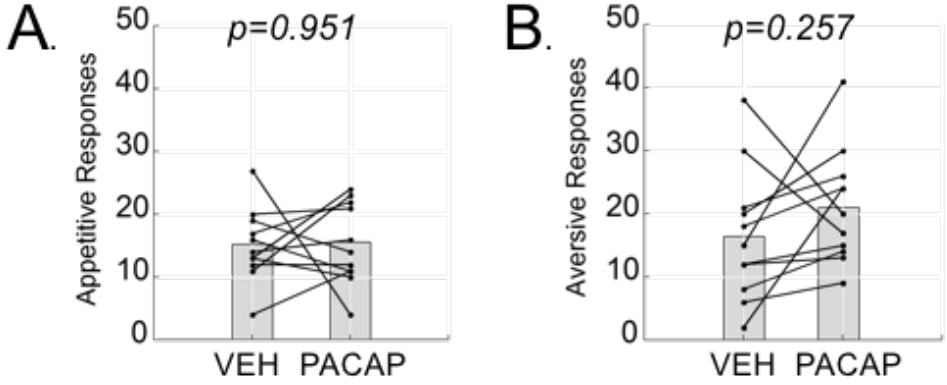


Figure 5.1 Acute administration of PACAP (50pmol/.25ul) into the VMN did not alter hedonic orofacial responses to a 1% sucrose solution. **(A)** A VMN PACAP microinjection 15 minutes prior to a taste reactivity test did not significantly alter appetitive responses (lateral tongue protrusions & paw licks) to the sucrose solution. **(B)** A VMN PACAP microinjection 15 minutes prior to a taste reactivity test did not significantly alter aversive responses (gapes & paw flails) to the sucrose solution. Data from each subject are presented, as well as the group mean. * = $p < .05$ compared to vehicle injection.

In an effort to identify potential PACAP afferents into the accumbens, we injected a 10% cholera toxin subunit B solution into the nucleus accumbens and then processed the tissue for *in situ* hybridization (Fig 5.3). We found that PACAP expressing cells in the prefrontal cortex project to the nucleus accumbens core (Fig 5.3C). Furthermore, a

separate experiment to label PAC1R mRNA's in the accumbens found that PAC1R was densely expressed in this region (Fig 5.4).

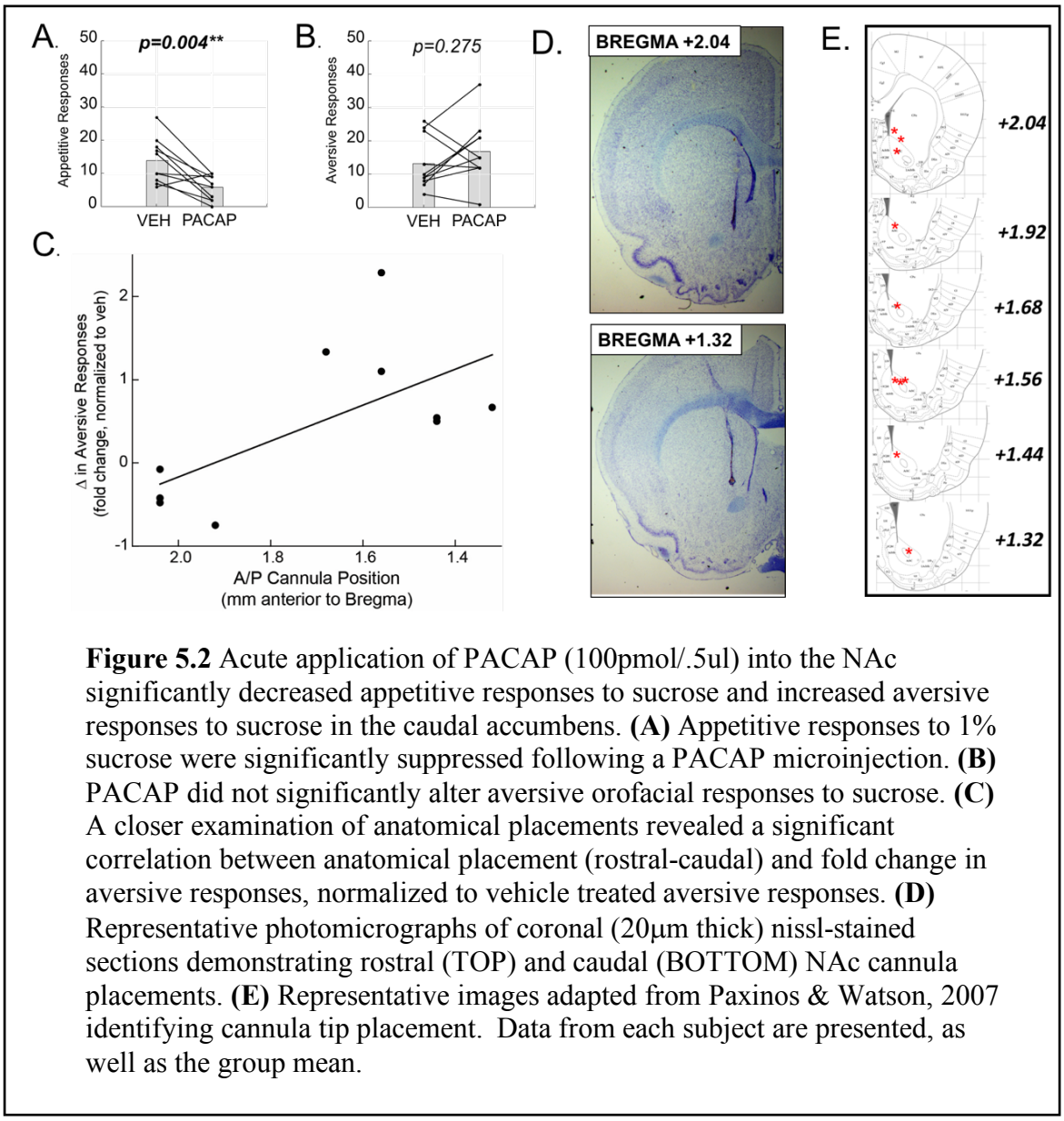


Figure 5.2 Acute application of PACAP (100pmol/.5ul) into the NAc significantly decreased appetitive responses to sucrose and increased aversive responses to sucrose in the caudal accumbens. **(A)** Appetitive responses to 1% sucrose were significantly suppressed following a PACAP microinjection. **(B)** PACAP did not significantly alter aversive orofacial responses to sucrose. **(C)** A closer examination of anatomical placements revealed a significant correlation between anatomical placement (rostral-caudal) and fold change in aversive responses, normalized to vehicle treated aversive responses. **(D)** Representative photomicrographs of coronal (20µm thick) nissl-stained sections demonstrating rostral (TOP) and caudal (BOTTOM) NAc cannula placements. **(E)** Representative images adapted from Paxinos & Watson, 2007 identifying cannula tip placement. Data from each subject are presented, as well as the group mean.

To assess if PACAP interacts with dopamine signaling in the accumbens, nucleus accumbens cannulated animals entrained to the two-meal binge eating paradigm were injected prior to meal 2 with either PACAP, or a D1-like receptor agonist (SKF 81297)

just prior to the PACAP microinjection (Fig 5.5). Interestingly, we found that animals pretreated with the D1 agonist, did not display PACAP-induced hypophagia for a palatable food (Fig 5.5; right; $P<0.05$).

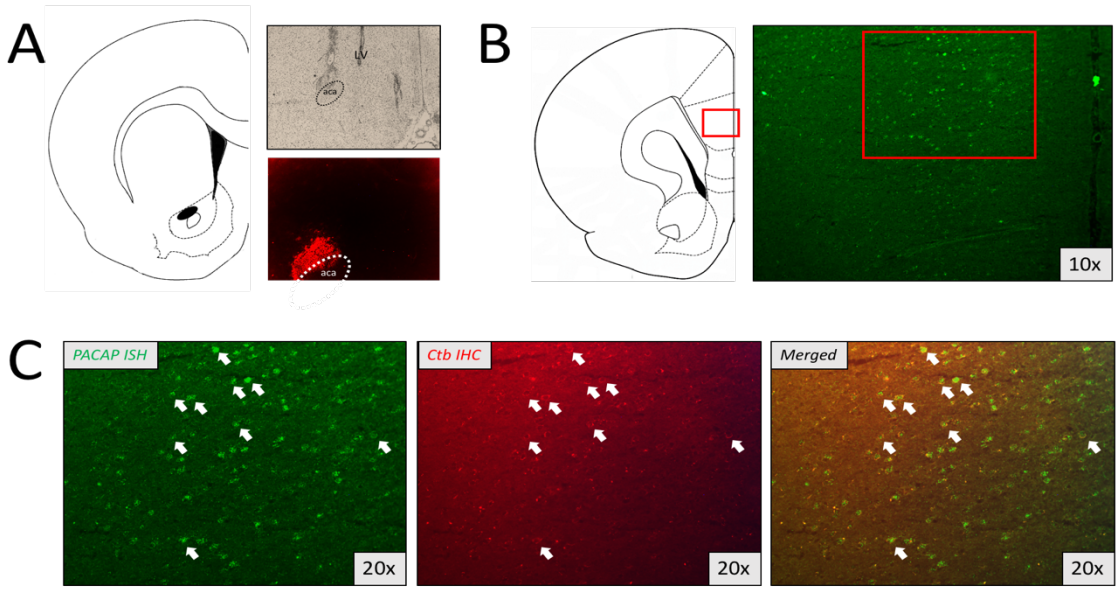


Figure 5.3 PACAP expressing nucleus accumbens core afferents arise from the prelimbic cortex. **(A)** Cannula placement showing where cholera toxin subunit-B was injected into the accumbens. **(B)** 10X image of PACAP mRNA expression in the prelimbic cortex. **(C)** 20X image showing co-labeling of prelimbic cells with PACAP mRNA and retrograde tracer.

With the evidence that PACAP increases system xc- activity and that system xc- is heavily implicated in other compulsive behaviors such as addiction (Kong et al., 2016), we examined if PACAP-induced decreases in palatable food consumption was dependent on system xc- activity (Fig 5.6). Identical to our previous studies, wildtype animals that received a PACAP microinjection prior to meal 2 consumed significantly less palatable diet, but system xc- KO rats did not respond to the PACAP microinjection. Interestingly,

both wildtype and KO's displayed similar hypophagic responses to a nucleus accumbens injection of baclofen and muscimol.

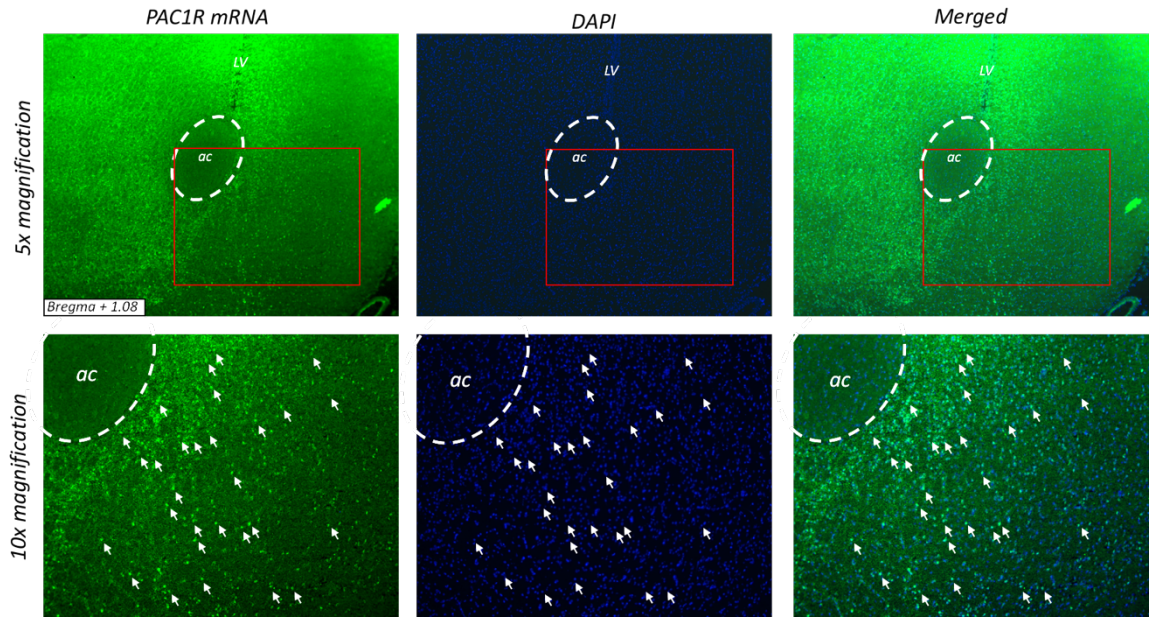


Figure 5.4 PAC1R mRNA is abundantly expressed in the rat nucleus accumbens core and shell region at 5X (top) and 10X (bottom) magnification.

DISCUSSION

With overconsumption as a major contributor to the obesity epidemic (Blundell & Cooling, 2000), it is clearly important to investigate the mechanisms by which eating *beyond satiety* is regulated. Previously, we reported that the neuropeptide PACAP acts in the rat NAc (but not the VMN) to specifically attenuate palatable food intake in satiated rats (Hurley et al., 2016a). To better characterize PACAP-induced voluntary reduction in palatable food intake, we used a rodent taste reactivity design to assess if PACAP microinjected into the nucleus accumbens (NAc) altered hedonic perceptions of sucrose.

In the first experiment, we found that PACAP microinjected into the VMN, an area where PACAP reduces feeding but *does not* affect palatable food consumption, had no effect on the expression of either positive (appetitive) or negative (aversive) responses to a 1% sucrose solution (Fig 5.1). This result was not unexpected since our prior work showed that PACAP administered in the VMN only attenuated hunger-driven feeding while having no effect on feeding driven by palatability (Hurley et al., 2016a). In contrast, administration of PACAP in the NAc significantly blunted appetitive responses to sucrose without altering the expression of aversive responses overall (Fig 5.2).

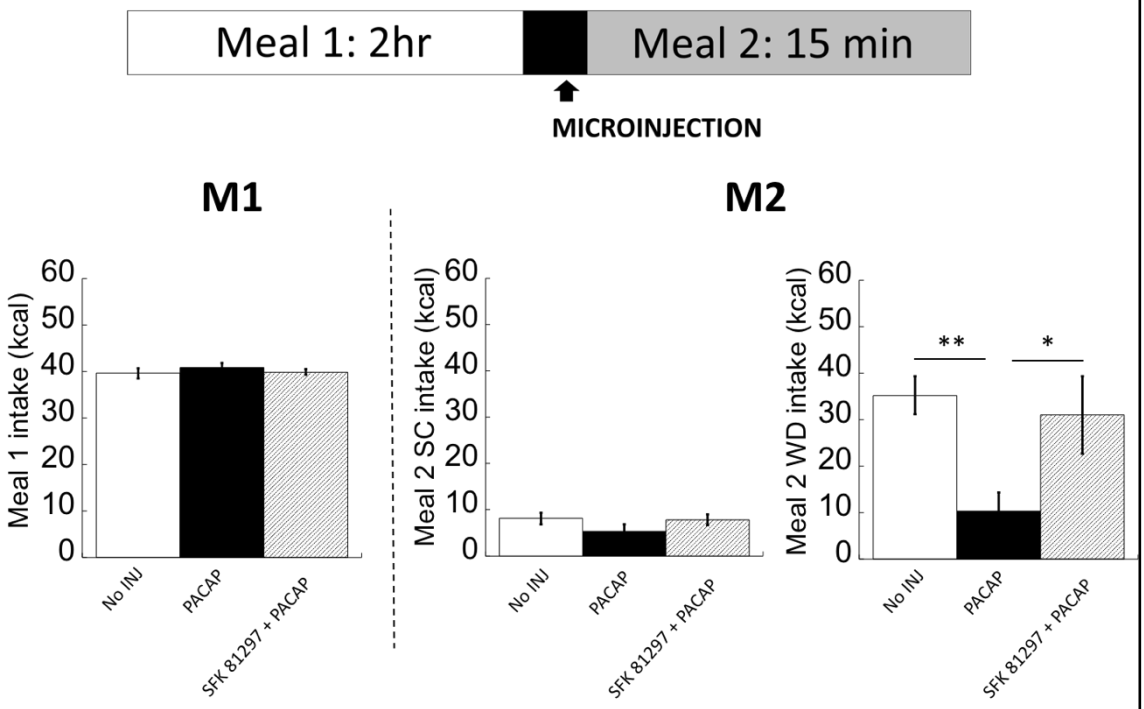


Figure 5.5 D1 agonist SKF 81297 occludes PACAP-induced hypophagia of palatable food in the nucleus accumbens core. **(Left)** Meal 1 SC intake from the three different injection days. **(Middle)** PACAP and SKF81297 + PACAP did not consume meal 2 SC differently than control. **(Right)** PACAP microinjection resulted in a significant reduction in western diet intake as compared to the no INJ group and pretreating animals with D1 agonist (SKF 81297) prevented PACAP induced hypophagia.

However, a closer examination of the anatomical placements of PACAP delivery revealed an additional layer of complexity in the NAc. By examining the behavioral responses along a rostral-caudal axis, we found that PACAP delivered to the rostral NAc decreased aversive responses to sucrose, while PACAP microinjected into the caudal NAc enhanced aversive responses (Fig 5.2C). Taken together, figure 5.1 and 5.2 emphasize an important point that the PACAP system differentially regulates behavior depending on the specific brain region.

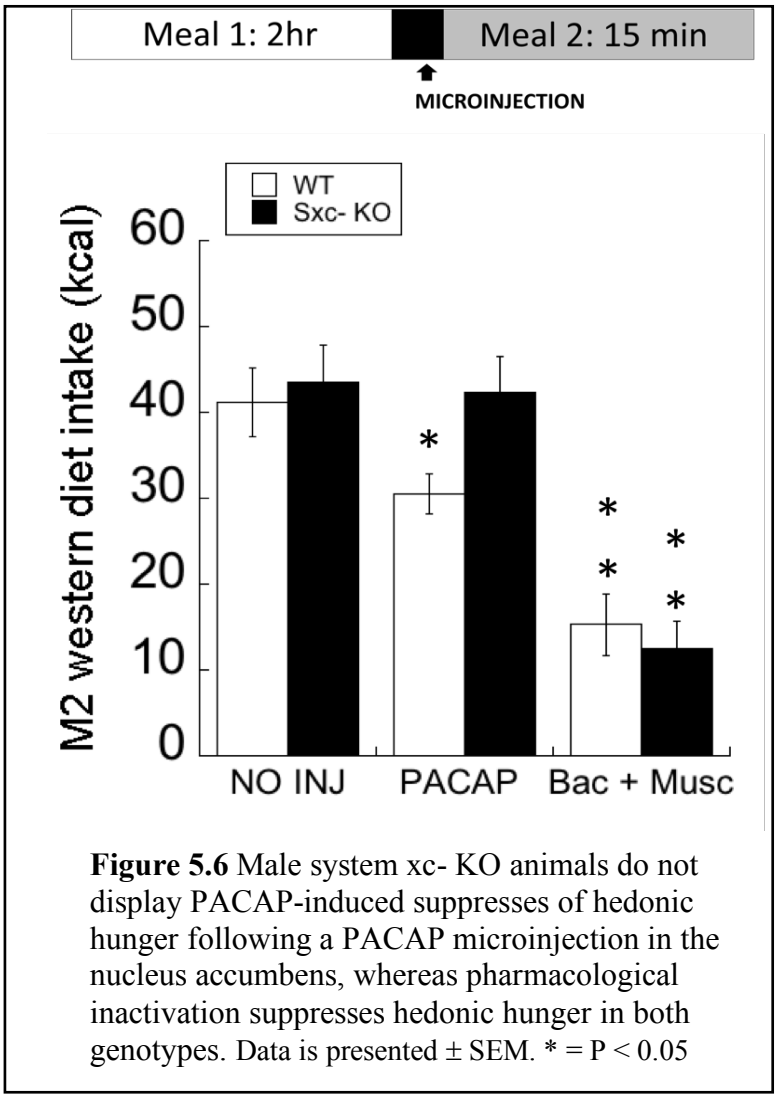


Figure 5.6 Male system xc- KO animals do not display PACAP-induced suppresses of hedonic hunger following a PACAP microinjection in the nucleus accumbens, whereas pharmacological inactivation suppresses hedonic hunger in both genotypes. Data is presented ± SEM. * = $P < 0.05$

PACAP enhancement of aversive taste reactivity in the caudal NAc is consistent with other studies demonstrating a similar NAc rostral-caudal gradient in regulating appetitive and aversive behaviors. For example, pharmacological inactivation of the caudal medial accumbens shell decreases food intake and appetitive responses to sucrose, while also increasing aversive responses (Ho & Berridge; Reynolds & Berridge, 2001). In contrast, inactivation of the rostral NAc stimulates feeding behavior and increases appetitive responses to sucrose while decreasing aversive responses.

These results further support our previous findings that bath application of PACAP in a slice preparation attenuates evoked activity in medium spiny neurons (Hurley et al., 2016a), suggesting that PACAP produces its region-specific effect on taste reactivity through inhibitory actions. It is important to recognize that the work identifying the rostrocaudal axis in the accumbens targeted the dorsomedial shell, whereas the current dataset targeted the area immediately adjacent to this site, in the core of the nucleus accumbens. A number of studies demonstrate that the core and the shell differentially regulate reward related behaviors (Floresco, McLaughlin, & Haluk, 2008; Ghitza, Fabbricatore, Prokopenko, & West, 2004) with a largely non-overlapping efferent distribution (Heimer, Zahm, Churchill, Kalivas, & Wohltmann, 1991). Thus, caution must be taken when applying what is known about the shell, to the core. Interestingly, emerging evidence demonstrates that the shell-core border, also known as the shore, makes connections with the lateral hypothalamus and importantly, these connections are not specific to the core or shell, rather they are specific to the dorsomedial component of the nucleus accumbens (Thompson & Swanson, 2010). Therefore, it is likely that our

pattern of data is similar to that of the Berridge lab, as both target the same cells in the dorsomedial aspect of the nucleus accumbens.

With our evidence that PACAP expressing cells in the prelimibic cortex project to abundant PAC1R expressing cells in the nucleus accumbens (Fig 5.3 and 5.4), it is notable that obese individuals have been reported to display hypoactivity in the prefrontal cortex (Small, 2009; van Meer, Charbonnier, & Smeets, 2016). Interestingly, obese individuals actively losing weight display enhanced medial prefrontal cortex activity when shown a cue for a food reward compared to obese individuals not actively dieting (Murdaugh, Cox, Cook, & Weller, 2012). As direct transcranial magnetic stimulation of the prefrontal cortex in obese individuals has been shown to decrease food intake and increase weight loss (Gluck et al., 2015), future studies will be necessary to identify the specific mechanisms by which prefrontal PACAP projections may regulate hedonic hunger in the nucleus accumbens.

As chemogenetic stimulation of D1-like receptor expressing cells in the accumbens increases feeding behavior and inhibition of D1-like receptor expressing medium spiny neurons decreases feeding behavior (Zhu et al., 2016), we determined if PACAP signaling in the nucleus accumbens interacts with dopaminergic signaling.

Our findings demonstrate that PACAP-induced hypophagia of palatable food in satiated rats is blocked by pretreatment of a D1 agonist (Fig 5.5). Anatomically, the pattern of PAC1R expression in the nucleus accumbens is such that it is not present in every cell, suggesting maybe it is only on specific subdivision of cells. Future studies are needed to determine the distribution of PAC1R on D1 and D2 cells.

In addition to potentially interacting with dopamine, our findings also suggest PACAP interacts with the cystine-glutamate antiporter, system xc-. Animals with a congenital deletion of system xc- do not display normal PACAP induced hypophagia of palatable food intake in satiated rats (Fig 5.6). However, system xc- knock out rats do positively respond to pharmacological inactivation using GABA agonists (Fig 5.6) suggesting that the circuitry that regulates palatable food consumption remains intact and functional. Taken together, PACAP, acting through system xc-, produces a net inhibitory effect on MSN's that regulate the neural encoding of reward.

Although PACAP regulates caloric deprivation induced feeding in the VMN, chapter V demonstrates that PACAP in the VMN does not regulate an animal's hedonic perception of a passively administered sucrose solution (Fig 5.1). By contrast, PACAP administered into highly discrete regions of the nucleus accumbens does greatly reduce the hedonic perception of the sucrose solution (Fig 5.2) illustrating that PACAP differentially and uniquely regulates various motivated feeding behaviors.

CHAPTER VI

GENERAL DISCUSSION

Summary

The overarching research goal of this dissertation was to assess the mechanisms by which the brain regulates feeding elicited *by distinct motivations*. Specifically, we examined the actions of the cystine prodrug, N-acetylcysteine (NAC; chapter II) and the neuropeptide pituitary adenylate cyclase activating polypeptide (PACAP) (chapter III-V) on differentially motivated feeding. This was achieved by using a variety of preclinical rodent feeding paradigms which were designed to elicit feeding of a single motive instead of multiple simultaneous motives. The two drives primarily studied in this thesis are feeding motivated by food deprivation or homeostatic hunger and feeding elicited by the palatability of the food rather than caloric need or hedonic hunger.

As binge eating disorder is thought to result, in part, through dysregulated hedonic hunger, chapter II utilized a well-accepted preclinical model of binge eating to study hedonic hunger. We found offering a male rat limited access to a highly palatable food adjunct to a nutritionally balance diet drives a compulsive state of rapid consumption, which emulates several characteristics of human binge eating disorder (Fig 2.1). As binge eating and substance abuse share a number of behavioral similarities (Fig 1.1), these two disorders may, in part, develop through similar maladaptive changes to the brain, such that one pharmacological intervention may be therapeutic to both disorders. To test this, we turn to the cystine pro-drug, N-acetylcysteine (NAC). Although originally used to treat acetaminophen overdose, emerging evidence in humans demonstrates that NAC

significantly suppresses compulsive behaviors such as obsessive-compulsive disorder, trichotillomania and substance abuse. As the effect of NAC on hedonic hunger-stimulated feeding had not yet been characterized, chapter II examines the impact systemic and central NAC manipulations have on different aspects of feeding behavior. Interestingly, systemic and central administration significantly diminishes binge feeding behavior while not effecting non-binge relating feeding and did not induce malaise (Fig 2.2, 2.3 & 2.4). A potential interpretation of these findings is NAC works centrally to specifically regulate feeding elicited by palatability or hedonic hunger, as feeding not specifically motivated toward palatability is unaffected by NAC manipulation.

It is important to note, animals maintained on the preclinical binge eating paradigm from chapter II display consistently steady, non-changing values of cumulative intake over the duration of the study (Fig 2.1A & Fig 2.3D). This is a result of animals increasing intake of the palatable food across experimental days, while also self-limiting consumption of the nutritionally balanced diet offered *ad lib* (Fig 2.1A). Taken together, when an animal maintained on this binge paradigm gains limited access to the highly palatable food source, the voracious feeding behavior observed is, in part, driven by hedonic hunger. However, as animals self-restrict *ad lib* food intake in anticipation of the palatable food to come, it is likely the feeding observed during the binge session may also be due to the need to fill caloric stores. Taken together, in chapter III we set out to develop a novel rodent binge eating model that would effectively separate homeostatic and hedonic hunger within the same animal.

In chapter III, we first determined 2HR daily access to chow is the optimal condition for producing a large meal stimulated by food deprivation (i.e. homeostatic

hunger) and ending in an extreme state of satiety. Animals with 3HR or 4HR access per day did not consume more calories than animals with only 2HR access (Fig 3.1B). Animals with 6HR per day access to chow, however, did eat significantly more than animals only given 2HR access (Fig 3.1B), but closer examination of the meal structure during 6 HR access revealed that after animals ate and become satiated they rested and then subsequently engaged in a second bout of feeding (Fig 3.1A). Additionally, human studies have used 22-HR food deprivation to drive "physical" or homeostatic-hunger (Lowe, Friedman, Mattes, Kopyt, & Gayda, 2000). Based on these results, we chose to investigate satiated animals (immediately following daily 2HR refeeding) who were then offered brief access (15 minutes) to more of the same diet, or the same diet with a novel flavor, which did produce any significant alteration of feeding behavior (Fig 3.2B & 3.2D). However, animals offered a high fat, highly palatable diet did engage in a large second bout of feeding (Fig 3.2A, 3.2B, 3.2D). Unlike the limited access model used in chapter II, the two-meal model did not produce any significant reduction in adjunct standard chow diet intake in animals expecting the limited palatable diet (Fig 3.2B). This results in significant increase in weight gain compared to control animals offered the same standard chow diet twice (Fig3.2C).

Previously, our lab has shown exogenous microinjection of pituitary adenylate cyclase-activating polypeptide (PACAP) into the ventromedial nucleus of the hypothalamus potently induces hypophagia as well as significant changes in metabolism (Resch et al., 2011; Resch et al., 2013; Resch, Maunze, et al., 2014). However, it was still unclear if exogenous PACAP injected into the VMN regulates homeostatic hunger, hedonic hunger or both. To address this, we entrained animals to the two-meal binge

paradigm (Fig 3.2) and then cannulated them for the VMN and conducted pharmacological manipulation. Interestingly, we found that exogenous PACAP delivered into the VMN regulated homeostatic hunger, while not effecting hedonic hunger induced feeding (Fig 3.3). Additionally, we found that the behavioral effect of PACAP was mimicked by pharmacological activation of cells in the VMN whereas pharmacological inactivation produces no significant effect to homeostatic or hedonic motivated feeding (Fig 3.3). Taken together, this suggests PACAP in the VMN may lead to the excitation of these neurons. Slice electrophysiological experiments conducted by our collaborator Dr. Qing-song Liu at the Medical College of Wisconsin confirms that bath application of PACAP in slice increases spontaneous firing of VMN neurons (Fig 3.5A). In addition to examining PACAP regulation of differentially motivated feeding in the VMN, we also targeted the nucleus accumbens in a similarly designed neuropharmacological experiment (Fig 3.4). We found exogenous PACAP microinjected into the nucleus accumbens had an opposite effect on feeding behavior as it suppressed hedonic hunger without affecting homeostatic hunger (Fig 3.4). Interestingly, this effect was mimicked in the accumbens by pharmacological inactivation, rather than activation, and confirmed in slice electrophysiological studies (Fig 3.5B). Taken together, the significance of the findings in chapter III demonstrate that the PACAP system does not just broadly diminish feeding behavior as we previously thought. Rather, depending on the site of action, PACAP regulates different aspects of feeding behavior through potentially divergent molecular mechanisms.

In chapter IV, we further investigate the mechanisms by which PACAP regulates homeostatic hunger in the VMN. Previous work demonstrates that antagonizing central

PAC1R signaling blocks central leptin-induced changes to feeding behavior and metabolism (Hawke et al., 2009). As it is not known whether this interaction is happening at the level of the VMN, in chapter IV we conducted both behavioral and molecular experiments designed to examine this question. We found antagonizing PAC1R signaling in the VMN prior to a VMN microinjection of leptin, blocked leptin-induced hypophagia, weight loss and changes to core body temperature (Fig 4.1). Using double *in situ* hybridization, we confirmed that PAC1R and the leptin receptor are co-expressed in VMN cells (Fig 4.2). In addition to behavioral and anatomical evidence for PACAP and leptin interaction in the VMN, we found PACAP microinjected into the VMN increases nuclear P-STAT3 accumulation (Fig 4.3A). This is significant because it is well documented that activation of the leptin receptor in the VMN drives the JAK2-STAT3 cascade (Frontini et al., 2008), which also results in the accumulation of nuclear P-STAT3 in the VMN. Finally, we demonstrated that both PACAP and leptin produce a significant increase in brain derived neurotrophic factor (BDNF) and suppressor of cytokine signaling 3 (SOCS3) mRNA three hours post VMN microinjection and this increase is blocked by pretreatment of PACAP 6-38 (Fig 4.4B). Taken together, PACAP regulates feeding in the VMN possibly through a common pathway shared with the hormone leptin.

In an effort to determine if PACAP-regulated feeding was only effective at suppressing homeostatic motivated feeding, we microinjected PACAP into the nucleus accumbens and surprisingly we found that exogenous PACAP also suppresses palatability-driven feeding (chapter III). Chapter V examines in greater detail the mechanisms by which PACAP regulates behavior in the nucleus accumbens.

Interestingly, we demonstrate that PACAP microinjected into the nucleus accumbens significantly reduces hedonic drive (Fig 5.2), which is specific to this brain region as PACAP microinjected into the VMN had no effect on the hedonic impact of a sweet tastant (Fig 5.1). Anatomically, we demonstrate that PAC1R is densely expressed in the ventral striatum (Fig 5.4), but not expressed in every cell. In an effort to identify the source of PACAP released in the nucleus accumbens, track tracing studies revealed that PACAP mRNA is expressed in the prelimbic cortex projects to the nucleus accumbens core (Fig 5.3). Additionally, we found pharmacological activation of D1-like receptors or global knockout of cysteine glutamate antiporter system xc- sufficient to block PACAP induced hypophagia in the accumbens, which suggests the PACAP-accumbens may be interact with or be dependent on these two mechanisms.

Why does the limited access model produce anticipatory self-restriction in SC, while the two-meal does not?

This thesis studied two preclinical rodent models of binge eating disorder: the limited access binge model and the two-meal model (Chapter II & III, V). Both models emulate several characteristics observed in human BED, namely a large bout of food intake of highly palatable food (Fig 2.1A; Fig 3.2B). The major difference between these two paradigms is the accessibility of SC in the limited access model and the restricted two-meal model. Over a 14-day period, animals entrained to the limited access model limit *ad lib* SC intake (which is available continuously), in anticipation of the highly palatable diet, which makes up a larger percentage of the daily kilocalorie intake with each successive binge session. This results in unchanging cumulative food intake and animals that do not gain weight or become obese (Corwin et al., 1998). Furthermore, this

self-restricting behavior indicates that the motivations to consume the highly palatable food were both hedonic and homeostatically driven. Interestingly, animals maintained on the two-meal paradigm do not display the self-restricting behavior observed in the limited access model. This is likely due to the fact that the feeding behavior in the limited access model involves motives working simultaneously to regulate feeding behavior, whereas the two-meal design temporally separates these drives, resulting in two isolated bouts of feeding (Fig 6.1).

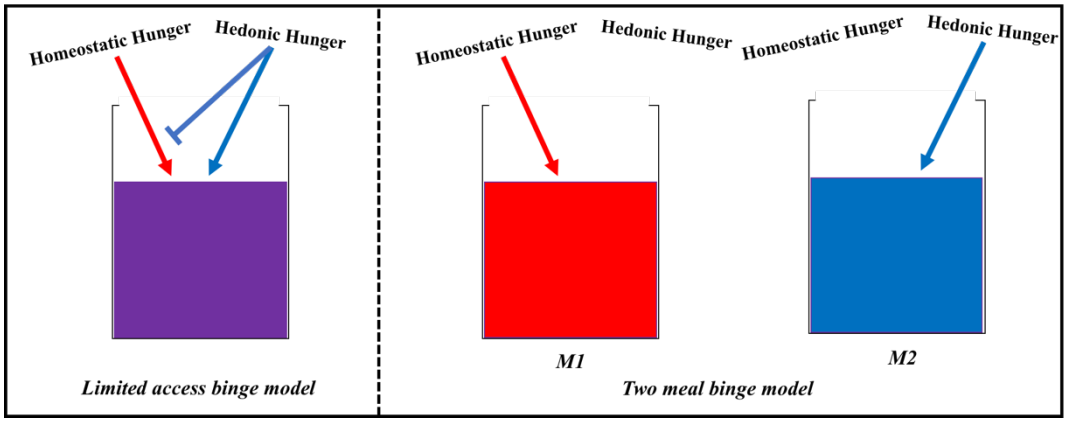


Figure 6.1 Diagram of feeding motives in the limited access and two-meal binge eating paradigm. M1= meal 1; M2= meal 2.

An alternative explanation for this difference in self-restricting behavior may be the daily food deprivation used in the two-meal paradigm. Food restriction is a useful tool to increase motivation in the field of behavioral neuroscience, as it is commonly used to facilitate self-administration of a number of drugs of abuse (Carroll, 1985; Carroll, France, & Meisch, 1979; de la Garza & Johanson, 1987). Increased motivation associated with food deprivation is likely occurring through changes in the mesolimbic signaling. Specifically, studies have identified that chronic food restriction decreases dopamine

transporter (DAT)-mediated reuptake of dopamine (Zhen, Reith, & Carr, 2006), increases DA receptors in the striatum (Lindblom et al., 2006) and increases excitability of midbrain dopamine cells (Branch et al., 2013). Although it is possible a hypersensitive dopamine system may protect against the self-restriction seen in undeprived animals; it should be noted that most of the studies identifying changes in mesolimbic dopamine signaling are using animals that are in a chronic negative energy state, whereas the two meal model animals are not in a negative energy state (Fig 3.2C). It would be interesting to determine if these changes are a result of the chronic negative energy state or cyclic food deprivation. Future studies will need to assess if two meal binge animals display similar adaptations in the mesolimbic dopamine circuitry.

What is the endogenous role for PACAP mediated signaling in the VMN?

To begin to answer this question we first need to first consider the typical feeding pattern of an *ad lib* fed rat. It is well known that Sprague-Dawley rats are nocturnal animals and, therefore, eat and are most active during the dark cycle, whereas during the light cycle animals eat very little and mostly sleep (Glendinning & Smith, 1994). Therefore, if endogenous PACAP released into the VMN acts to induce hypophagia, it is unlikely PACAP is being released into the VMN during the dark cycle as the animals are actively consuming throughout this period. What is more likely is that PACAP signaling in the VMN is important for the maintenance of light cycle feeding behavior as animals during the light cycle engage in little to no feeding behavior. In figure 4.1, it is clear that blockade of PAC1R dependent signaling in the VMN at the onset of the dark cycle, produces no significant effect on feeding behavior, which is consistent with work

previously published by our laboratory (Resch et al., 2011). In rats microinjected in the VMN during the middle of the light cycle with either vehicle, a low, or a high dose of PACAP 6-38 there was a significant dose-dependent increase in feeding behavior (Fig 6.2). Importantly, the high dose is the same dose that produces no significant changes in feeding behavior when injected during the dark cycle. This data suggests endogenous PAC1R dependent signaling occurs primarily during the light cycle perhaps to inhibit feeding during this time period, whereas dark phase receptor blockade had no significant effect on feeding because endogenous PACAP signaling may be minimal at this time of day (Resch et al., 2011).

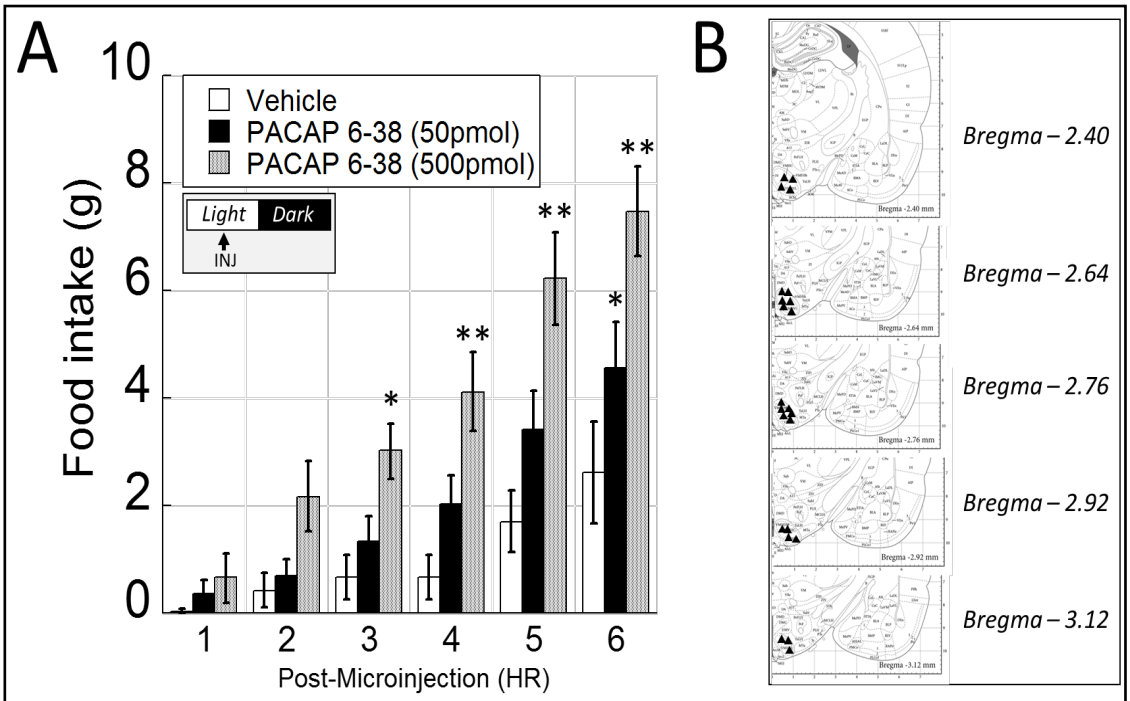


Figure 6.2 Preliminary evidence suggesting PAC1R dependent signaling in the VMN crucial for maintaining normal light cycle fasting. **(A)** VMN cannulated animals (n=5-7/group/0.25µl/side) injected with PACAP 6-38 consumed significantly more calories than vehicle. **(B)** Cannula placements considered VMN hits. Data presented ± SEM, Tx $F(2,214)= 8.130$, $p=0.001$; Tx X Time $F(9, 214)= 4.578$, $p < 0.001$* or **= $p<0.05$.

Interestingly, studies have demonstrated *in vitro* that PACAP, both 1-38 and 1-27 can be N-terminally truncated to produce PACAP receptor antagonizing metabolites by dipeptidyl peptidase IV (Green, Irwin, & Flatt, 2006; L. Zhu et al., 2003). DPP4 metabolizes PACAP 1-27 at three different sites and administration of DPP4 metabolites of PACAP1-27, blocks PACAP induced increase in plasma glucose levels (Green et al., 2006). An alternative explanation for the pattern of data observed in our light-dark PACAP 6-38 data is that PACAP is tonically released, but enzymatically truncated into PACAP 6-38 during the dark cycle by DPP4. This is an interesting idea that DPP4 could be mediating a PACAP negative feedback loop, however little is known about DPP4 having action in the brain.

What role does BDNF play in leptin & PACAP regulation of feeding in the VMN?

Interestingly, brain derived neurotrophic factor or BDNF mRNA levels in the VMN are highly sensitive to energy state, which suggests BDNF action may be important for regulating homeostatic hunger related processes (Conner et al., 1997; Xu et al., 2003). Further evidence of this is BDNF delivered centrally (Pelleymounter, Cullen, & Wellman, 1995), or directly into the VMN display significant suppression in feeding behavior (Wang et al., 2010; Wang, Bomberg, Levine, Billington, & Kotz, 2007). Furthermore, a global or VMN-specific knockout leads to hyperphagia and increased weight gain (Lyons et al., 1999; Mou et al., 2015; Vanderklish & Edelman, 2002). Finally, knockdown of BDNF disrupts VMN-leptin induced hypophagia (Lindblom et al., 2006). With PACAP and leptin both increasing BDNF mRNA (Fig 4.3B) and the abundant co-localization of PAC1R and BDNF in the VMN (Fig 4.2), we hypothesize

that PACAP and leptin-induced effects on feeding and metabolism are dependent on BDNF as the main effector system. Future studies will need to knockdown BDNF in the VMN and microinject PACAP to see if, like leptin, PACAP induced hypophagia is impaired.

Why would two peptides (PACAP & leptin) work to produce the same effect?

PACAP and leptin manipulations in the ventromedial nucleus of the hypothalamus produce a number of similar changes to energy expenditure (Choi et al., 1999; Dhillon et al., 2006; Jacob et al., 1997; Resch et al., 2011; Resch et al., 2013). Chapter IV demonstrates disrupting endogenous PAC1R dependent signaling in the VMN is sufficient to block leptin induced behavioral and molecular changes. Furthermore, *in situ* hybridization revealed that both PAC1R and the leptin receptor are co-localized in the VMN, which suggests there may be some form of interaction at the molecular level.

We hypothesize leptin and PACAP signaling in the VMN are not redundant, rather each peptide serve a purpose in reporting different aspects of energy status. As leptin is secreted peripherally by fat cells and does not vary greatly over days or in response to acute caloric intake, leptin serves to report the long-term energy status of the organism. Whereas central PACAP may work to report acute energy status as it relates to homeostatic hunger. Collectively, the combination of peripheral and central markers of energy state increases the complexity by which feeding behavior is regulated in the VMN. To determine if PACAP and leptin work to regulate a common pathway in the

VMN, future studies will have to block the leptin receptor and test if PACAP is still able to induce changes in food intake and metabolism.

Frontostriatal PACAP dysregulation in the development of binge eating disorder.

As PACAP infused into the nucleus accumbens, suppresses palatability driven feeding (chapter III) and hedonic drive (chapter V), it is becoming increasingly important to identify the relevant PACAP afferents into this region. The nucleus accumbens core receives a number of glutamatergic afferents primarily from the prelimbic cortex (PL), the basolateral amygdala (BLA) and the ventral subiculum of the hippocampus (Britt et al., 2012). In chapter V, we identified PACAP mRNA expressing neurons in the PL project to the core (Fig 5.3). As electric stimulation of the PL, decreases binge eating in rodents (Sarica et al., 2018), and pharmacological inactivation of the PL specifically increases binge eating in rodents (Corwin et al., 2016), the prelimbic cortex appears to be a key structure in regulating binge eating. Interestingly, reinstatement of cocaine seeking causes the prelimbic cortex to become active (Kufahl et al., 2009). As neuropeptides are typically only released under high frequency stimulation (Smith & Eiden, 2012), we hypothesize that motivated behavior towards a natural or unnatural reward will result in repeated firing of these prelimbic-core glutamatergic neurons, but after a certain period of time, once high frequency stimulation is achieved, we expect PACAP to be co-released with glutamate and effectively inhibits the seeking of the reward. Therefore, in BED where human and rodent models display significant signs of prefrontal cortex dysfunction (Aloi et al., 2015; Balodis et al., 2013; Chawla et al., 2017), it is possible high frequency

stimulation is never achieved so therefore, there is no PACAP-induced 'brake' on the reward consumption. To test this, future studies should stimulate the prelimbic cortex following a PACAP 6-38 microinjection into the nucleus accumbens core to determine if PL stimulation is still effective at suppressing binge eating in rats.

What is the physiological importance of differentially motivated feeding?

In chapter III, we demonstrate the neuropeptide PACAP microinjected in the VMN only disrupts feeding elicited by food deprivation (i.e. homeostatic hunger), while PACAP microinjected into the same region was ineffective at suppressing feeding driven by palatability (hedonic hunger). These results indicate endogenous neuronal activity in the VMN, *during feeding motivated by food deprivation*, is critical for driving this behavior as exogenous PACAP application disrupts this specific type of motivated feeding. However, when caloric stores have been filled and feeding is motivated by the hedonic value of the food rather than the caloric need, we see PACAP, AMPA and bacelofen+musicmol manipulations in the VMN do not disrupt hedonic hunger driven feeding, suggesting activity in the VMN is not critical for driving this type of hunger.

These findings demonstrate the different "motives" we assign as the reasons we engage in feeding, are driven in part by unique patterns of neuronal recruitment involving different brain regions and signaling systems that govern discrete aspects of feeding behavior. Evolution has shaped the mammalian brain in such a manner that feeding can be stimulated and regulated through a number of diverging, complex mechanisms that are not purely dependent on energy state. This is physiologically important, because the ability to consume and seek calories when food deprived or when presented with an

energy dense palatable food insures that the organism continues to survive and propagate genetic material. Interestingly, in the nucleus accumbens, we observed PACAP was only effective at suppressing feeding driven by hedonic hunger while being ineffective at blocking homeostatic hunger. Our results add a layer of complexity to this as we demonstrate the PACAP system, which was previously thought to regulate *all* forms of feeding, regulates different aspects of feeding behavior, dependent on the region of the brain.

Conclusion

In 2012, a German research group conducted a survey probing subjects to explain why they eat what they eat and the research group determined that there were 331 unique "motives" people attributed to driving consumption (Renner, Sproesser, Strohbach, & Schupp, 2012). Of these motives, the most frequent were caloric driven feeding or homeostatic hunger, and palatability driven-feeding or hedonic hunger. Each motivated desire to eat (i.e. hunger) is the culmination of a certain pattern of brain activity that are recruited by both internal and external cues (sensory, blood glucose, stomach distension, gut-brain peptides, etc.). These motives (i.e. patterns of activation) *do not* regulate feeding in isolation but rather work in concert to guide intake. The complex manner by which the brain regulates the nuances of feeding behavior makes it extremely difficult to study where disorders in feeding manifest. In an effort to understand how the brain differentially regulates these motivated feeding states, the current dissertation employed a number of preclinical rodent paradigms (*ad lib* fed SC, restricted SC, limited access binge, two meal binge) designed to elicit certain types of feeding. From this we learned

that therapeutic approaches to treat substance abuse and other compulsive behaviors, can be effective at curbing binge eating as the compulsive aspect of these disorders likely manifests through a common route. Additionally, we demonstrated that the neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP) *does not* regulate feeding in the same manner all over the brain, which is highly novel. We observed that PACAP in the hypothalamus, suppressed homeostatic hunger by interacting with leptin and BDNF, while PACAP microinjected into the striatum, suppresses a fundamentally different motivated feeding state (i.e. hedonic hunger) while also impacting hedonic perception. In conclusion, the findings presented here shed light on the complexities of the PACAP system in the regulation of feeding behavior.

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